Biodiversity and ecophysiology of aeroterrestrial green algae (Trebouxiophyceae, Chlorophyta)

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Summary

Aeroterrestrial algae typically colonize the interface between lithosphere and atmosphere, forming dense biofilms on natural and artificial substrata. Algal biofilms are a widespread and sometimes unwelcome phenomenon, as they cause discolorations and accelerate weathering of anthropogenic surfaces due to co-occurring (possibly harmful) bacteria and fungi. Thus, aeroterrestrial algae attract scientific interest from an applied *and* ecological point of view. The present study focused on the biodiversity and ecophysiology of aeroterrestrial green algae from mid latitudes (mainly Germany). Culture-independent phylogenetic analysis by project co-workers (Mudimu 2008) revealed detailed knowledge about species composition in aeroterrestrial communities, identifying *Apatococcus* spp. as the most abundant organisms followed by *Chloroidium*, *Stichococcus* and *Coccomyxa* spp. (Trebouxiophyceae, Chlorophyta). The taxonomical status of aeroterrestrial abundant genus *Chloroidium* (formerly assigned to *"Chlorella"*) was clarified by a multiphasic approach combining molecular, morphological and physiological characters. The systematical screening for low molecular weight carbohydrates in diverse phylogenetical groups revealed different distribution pattern of polyols, which are otherwise uncommon in Chlorophyta. The value of polyols for chemotaxonomical applications (combining phylogeny with physiology) is considered as high. However, the emphasis of this study was the evaluation of major environmental factors controlling growth and reproduction of aeroterrestrial algae, as they are subject to multiple and combined abiotic stresses in their challenging environment. Optimum growth curves concerning various environmental factors were recorded, characterizing aeroterrestrial green algae as euryoecious. Investigations focused on growth under water limitation, as this factor is considered a prerequisite for the aeroterrestrial way of life. The data indicate a high tolerance of aeroterrestrial green algae against low water availability, which is partly based on the biochemical capability to synthesize and accumulate "water-holding" polyols. Moreover, their function as organic osmolytes was demonstrated. The discrepancy between the dominance of *Apatococcus lobatus* in aeroterrestrial biofilms and its minor competitive strength in terms of growth rate under culture conditions led to investigations about mixotrophy in this species. Mixotrophy enables algae to use organic substrates as carbon- and energy-sources, thus acting act different trophic levels within the ecosystem. Using a microsensor, the strong attenuation of ambient light was measured inside natural and artificial (pure *Apatococcus*) biofilms. As radiation is a limiting factor in deeper biofilm layers, mixotrophy is assumed as a competitive advantage against faster dividing algal taxa in the top layer.

Zusammenfassung

Aeroterrestrische Algen besiedeln die Grenzfläche zwischen Atmosphäre und Lithosphäre, wo sie dichte Biofilme auf natürlichen und artifiziellen Substraten bilden. Grüne Biofilme sind weitverbreitet und häufig unerwünscht, da sie anthropogene Substrate verfärben und zu deren Verwitterung beitragen sowie einen Nährboden für (möglicherweise gesundheitsschädigende) Bakterien und Pilze bieten. Daher sind sie vom angewandten *und* wissenschaftlichen Standpunkt interessant. Die vorliegende Arbeit beschäftigt sich mit der Biodiversität und der Ökophysiologie aeroterrestrischer Grünalgen mittlerer Breiten (hauptsächlich Deutschland). Kulturunabhängige phylogenetische Analysen durch die Projektpartner (Mudimu 2008) konnten die Artzusammensetzung in aeroterrestrischen Gemeinschaften ausführlich aufklären und identifizierten *Apatococcus* spp. als abundanteste Vertreter, gefolgt von *Chloroidium*, *Stichococcus* und *Coccomyxa* spp. (Trebouxiopyceae, Chlorophyta). Der taxonomische Status häufig vorkommender *Chloroidium* spp. (bisher „*Chlorella*" zugeordnet) wurde durch die Kombination molekularer, morphologischer und physiologischer Merkmale innerhalb eines multiphasischen Ansatzes umfassend aufgeklärt. Das systematische Screening nach niedrig-molekularen Kohlenwasserstoffen in diversen phylogenetischen Gruppen ergab unterschiedliche Verteilungsmuster von Polyolen, deren Vorkommen für Chlorophyta ungewöhnlich ist. Die Bedeutung von Polyolen für chemotaxonomische Anwendungen, welche Phylogenie mit Physiologie verknüpfen, ist daher als hoch einzuschätzen. Der Schwerpunkt der vorliegenden Arbeit liegt in der Bewertung von Schlüsselfaktoren für Wachstum und Reproduktion aeroterrestrischer Algen, da ihr Lebensraum durch die Kombination verschiedener Stressfaktoren gekennzeichnet ist. Optimum-Wachstumskurven in Abhängigkeit verschiedener Umweltfaktoren charakterisierten aeroterrestrische Grünalgen als euryök. Der Fokus der Untersuchungen lag auf dem Faktor Wasserverfügbarkeit, da dieser als Grundvoraussetzung für die aeroterrestrische Lebensweise diskutiert wird, bisher aber kaum untersucht wurde. Die Toleranz der untersuchten Isolate gegenüber Wasserlimitation gründet teilweise auf der Akkumulation von Polyolen, deren Funktion als organische Osmolyte nachgewiesen wurde. Die Diskrepanz zwischen der Dominanz von *Apatococcus lobatus* in aeroterrestrischen Biofilmen und seiner geringen Konkurrenzstärke bezüglich der Wachstumsgeschwindigkeit führte zu Untersuchungen über Mixotrophie in diesem Organismus. Mixotrophie befähigt Algen, organische Substrate als Kohlenstoff- und Energiequelle zu verwerten und so im Ökosystem auf unterschiedlichen trophischen Ebenen zu agieren. Mithilfe eines Mikrosensors konnte die starke Licht-Attenuation innerhalb natürlicher und artifizieller (*Apatococcus* Reinkulturen) Biofilme gemessen werden. Da Strahlung ein limitierender Faktor in tieferen Biofilm-Schichten ist, wird Mixotrophie hier als ein Wettbewerbsvorteil gegenüber schnellwachsenden autotrophen Algen in lichtgesättigten Schichten bewertet.

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*Chapters 3.1, 3.3 and 3.4 are only cited within the results-section, as the authors' copy-right agreements with respective journals do not allow an additional online publication.

1. General Introduction

The phenomenon – definition and occurrence of aeroterrestrial biofilms

Biofilms occur at all scales and in virtually all environments. Their size can range from single monospecies cell layers to several centimetres thick microbial mats consisting of autotrophic and heterotrophic partners, with motile species moving through the matrix and grazers on its surface. In general, life in biofilms represents an enhanced protection against environmental stress, the possibility of specialized metabolic performance with mutualistic or symbiotic lifestyles and a defence strategy against competitors or grazers. Substrata hosting biofilms are usually submerged in or exposed to an aqueous environment.

Aeroterrestrial biofilms which are subject of this study are located at the atmosphere-lithosphere interface and are, thus, not permanently supplied with water. Additionally, these habitats are characterized by wide temperature and radiation amplitudes. Per definition, biofilms form "heterogeneous matrices of microorganisms held together and tightly bound to underlying surfaces by extracellular polymeric substances (EPS) that develop when nutrients from the surrounding environment are available" (Rosenberg 1989). Usually, aeroterrestrial biofilms consist of autotrophic algae or cyanobacteria, heterotrophic bacteria, and fungi (Gorbushina and Broughton 2009) in different proportions. Lichen symbiosis constitutes a special version of aeroterrestrial microorganisms, as the symbiosis between fungus and photosynthetic partner is very close and advantageous in terms of survival and competition in extreme environments. Lichen associations are known as primary colonizers of habitats lacking soil (unfavourable for higher plants) and constitute the sole vegetation in e.g. high latitudes (Nash 2008). Macroscopic appearance of biofilms (green, red, brown or black colouring) is determined by pigment composition of the dominating species, whose occurrence in turn depends on the geographical site and microclimatic features of the habitat. Extreme environments as hot deserts (Lewis and Flechtner 2004, Büdel et al. 2009), Polar Regions (Broady 1986), high mountains and acidic mining sites (Lukesova 2001) are colonized as well as more "comfortable" substrata such as tree barks and anthropogenic surfaces in all climatic zones (Ettl and Gärtner 1995, Tomaselli et al. 2000, Rindi and Guiry 2002, Barberousse et al. 2006). Aeroterrestrial algae are perhaps the most obvious, and most overlooked, group of algae as non-phycologists tend to dismiss them as nuisance and phycologists as minor variants of more important aquatic forms (Nienow 1996). Colonization of terrestrial surfaces proceeds by settling of aeroplanktonic microorganisms as bacteria, fungi, algal propagules and resting

spores which are transported by wind, rain water and migrating animals (Schlichting et al. 1978, Marshal and Chalmers 1997, Tormo et al. 2001, Schumann et al. 2004, Sharma et al. 2007 and references therein). Successful establishment of the deposited organisms depends on local environmental conditions and the ability to attach rapidly and strongly to a substrate (Karsten et al. 2007, Mostaert et al. 2009). On anthropogenic substrates, such as walls, fences and roof tiles, biofilms have already been recognized in the beginning of the nineteenth century, but most studies have focused on the biofouling aspect rather than their diversity and ecology (Rindi 2007 and references therein). Biofilms and concomitant EPS slowly change the substrate including the physical stability, the surface pH and the hydrophobicity (Gorbushina 2007 and references therein). Such biocorrosion proceeds by growth of cells inside the substrate (endolithic) or by subsequent freezing (swelling) and thawing (shrinking) of overlying biomass, resulting in erosion of the substrate surface by mechanical action. Both processes cause the separation of particles (Warscheid et al. 1991, Gaylarde and Morton 1999) and thus weathering of the substrate. Additionally, the chemical impact of respiratory CO_2, H^+ and organic acids causes dissolution of substrates, particularly of calcareous ones (Gorbushina and Broughton 2009, Hoppert et al. 2004). However, these processes are mainly associated with heterotrophic organisms such as co-occurring bacteria and fungi. Furthermore, decay of algal biofilms by fungi and bacteria may cause harmful health reactions such as asthma or allergies. Therefore, many reports about aeroterrestrial biofilms monitor growth and biofouling activity on urban buildings, churches, historical palaces and cultural monuments (Flores et al. 1997, Gaylarde et al. 2001, Darienko and Hoffmann 2003, Zurita et al. 2005, Rindi 2007).

The aeroterrestrial habitat

In general, algae are regarded as aquatic organisms and only a small proportion of the known species lives in terrestrial habitats (Lee 1999). It is not clear how many transitions to land have taken place, but it has been shown that terrestrial members occur in at least six groups: Trebouxiophyceae, Chlorophyceae, Ulvophyceae, Chlorokybophyceae, Klebsormidiophyceae and Zygnemophyceae (Rindi 2010 and references therein). Aeroterrestrial microorganisms are exposed to harsher and more variable environmental conditions than their aquatic counterparts (for review see Karsten et al. 2007, Gorbushina and Broughton 2009), where the surrounding water usually buffers abrupt changes of radiation (PAR and UVR) and temperature. The most important feature of any aeroterrestrial habitat is *water availability* (Turner 1975, Nienow 1996, Häubner 2006, Gladis and Schumann (submitted)), and water restriction is the most critical stressor affecting growth and survival of aeroterrestrial biofilms (Chang et al. 2007). Water availability in terrestrial environments can fluctuate from short to long time scales depending on

the specific habitat. Due to rain, snow or condensed water droplets, aeroterrestrial algal biofilms can be water-saturated, periodically water-limited or even water-deficient (desiccation or freezing). The frequency and intensity of water supply fluctuates strongly in diurnal and seasonal rhythms and is dependent on geographical location, while the storage of water depends additionally on microclimatic factors, the shape of *substratum* and the amount of EPS excreted by the microorganisms. A strict substratum-specificity has been reported for several species and locations (Schlichting 1975, Tiano et al. 1995, Rindi and Guiry 2002, Crispim et al. 2003). In general, high porosity and the resulting water holding capacity favor microalgal growth (Gladis and Schumann 2010). Nevertheless, aeroterrestrial biofilms occur on all kinds of smooth and coarse substrates and if sufficient water is available to support growth and survival, the substrate plays a minor role. The persistence of water in a habitat depends on evaporation rate which in turn is coupled with air temperature, strength and direction of prevailing winds and degree of radiation. Mild climate and adjacent vegetation support algal growth on buildings. In the northern hemisphere the so called weather (northern) side of buildings is stronger infested by algae than southern oriented facades (Karsten et al. 2005, Barberousse et al. 2006, own observations). Some coastal regions are strongly influenced by salt spray (e.g. Ireland) and aeroterrestrial microorganisms face physiological drought caused by a saline environment. On the other hand, high salinities can be derived from anthropogenic substrata as historical buildings (e.g. nitrate: Bretschneider 2007). However, s*alinity* is an uncommon stressor in the aeroterrestrial habitat, although the protective mechanisms towards water restriction and osmotic stress are comparable (for detailed discussion see chapter 3.4). Depending on latitude and geographical conditions, the level of *radiation* on earth fluctuates between excessive, moderate and scarce (for photosynthetic activity). On an annual average, the poles receive least irradiance while at equatorial regions solar radiation impacts at a 90° angle resulting in a maximal radiation budget. The maximum solar irradiance on Earth at local noon is 2374 µmol $m^{-2}s^{-1}$ (Mobley 1994). The spectrum of solar radiation ranges from the ultraviolet (ultra violet radiation (UVR): 100-400 nm) to the visible (photosynthetically active radiation (PAR): 400-700 nm) and infrared (>700 nm) region. Particularly UVB (280-315 nm) has strong mutagenic effects on algae (Karsten 2008). While the effects of radiation are moderate in aquatic systems and decrease exponentially with depth and increasing turbidity and concentration of yellow substances (Kirk 1994), aeroterrestrial algal assemblages are usually stronger exposed. The synthesis of UV-sunscreen compounds in aeroterrestrial algae, lichens and fungi is well documented as effective photoprotective strategy (Büdel et al. 1997, Reisser and Houben 2001, Kogej et al. 2006, Karsten et al. 2007). While water availability is regarded as key factor for terrestrial habitats, light availability is the limiting factor in aquatic ecosystems, as the photic zone is minute in contrast to the total water column (Dubinsky and Schofield

2009). The range of *temperature* on top of the lithosphere is significantly higher than in the ambient atmosphere and hence represents another considerable stressor. On bare terrestrial rock surfaces temperature can range between -45°C and +60°C (Gorbushina 2007), while roof tops can even reach a maximal temperature of 85°C (Potts 1994). However, some terrestrial habitats as e.g. endolithic ecosystems are buffered against extreme temperature fluctuations (e.g. underneath alpine soil crusts (Karsten, pers. communication, Walker and Pace 2007). The exact temperature regime of any particular site depends on the amount of solar radiation reaching the surface, which in turn is a function of latitude, elevation, orientation, degree of shading and reflectance of the substratum. Due to the high heat storage capacity of water, seasonal and diurnal atmospheric temperature fluctuations are well buffered in aquatic habitats, creating a thermally stable environment. The global *air circulation* system is very complex and again a function of latitudinal solar heat input and geographical conditions. Wind is mainly responsible for the atmospheric distribution of microorganisms. It erodes and removes established biofilms but on the other hand supplies new inoculum to barren substrata, thus contributing to the cosmopolitan distribution of many microorganisms (Nienow 1996, Gorbushina et al. 2007, Gorbushina and Broughton 2009). A prominent wind system transporting dust (with attached microorganisms) from the African continent to south Europe is the Scirocco. A negative effect of wind on aeroterrestrial biofilms is an increased evaporation rate, reducing the availability of water to the organisms. Therefore, it is commonly observed that green algal biofilms grow preferential on the leeward side of trees and stones (Barkman 1958, Broady 1986). The *nutrient supply* of aeroterrestrial biofilms is still a matter of debate and probably as irregular as other abiotic factors (Karsten et al. 2007). Nutrients are transported by rain water and snow (e.g. Karsten et al. 2005), aerosols, dust or soil particles (Gorbushina 2007). Sources of such atmospheric (external) nutrients are agricultural land use, anthropogenic sewage and industrial emissions (Schumann et al. 2004). Remineralization of dead algal cells, EPS and photosynthetic products inside aeroterrestrial biofilms by heterotrophic organisms is regarded as internal nutrient source (Karsten et al. 2007). In addition, some building materials (e.g. mineral plaster) are nutrient rich.

Algae

Algae are not a taxonomical entity in terms of phylogeny or systematics, they rather represent an ecological unit due to their major distribution in aquatic habitats and their ability to carry out photosynthesis. The name originates from colloquial language and summarizes a group of largely uniform but in particular very different organisms. Algae are a large and diverse group of eukaryotic photosynthetic organisms occurring in almost every habitat. They exhibit an impressive morphological

diversity, ranging from tiny unicells to huge kelps over 50 m long. Initially, the term alga was used for macroscopic marine plants, as seaweeds, and has been extended to microscopically photosynthetic organisms (microalgae). In contrast to higher plants *microalgae* mainly lack functional differentiation. Their biodiversity is largely under-investigated and is estimated to range from 200,000, up to more than 1,000,000 species (Norton et al. 1996). Nevertheless, estimations of "real" species numbers are extremely vague because of the pending situation in species concepts and the existence of many undiscovered cryptic species (Coesel and Krientiz 2008).

The first algal groups arose between 1 and 1.5 billion years ago (Douzery et al. 2004, Yoon et al. 2004) after the ingestion and retention (endosymbiosis) of a photosynthetic cyanobacterium within a heterotrophic eukaryotic organism (Raven 1997). This event gave rise to the primary plastids which are still present in the Glaucophyta, red and green algal lineages including land plants (Reyes-Prieto et al. 2007). These three lineages are collectively called Plantae or Archaeplastida (Cavalier-Smith 1981, Adl et al. 2005). The other photosynthetic protists arose through secondary endosymbiosis of either a green or a red alga. The euglenids and chlorarachniophytes are thought to have acquired their plastids from a green alga in two separate secondary endosymbiotic events, while molecular evidence suggests that the red algal plastid of cryptomonads, heterokonts, haptophytes, apicomplexans and dinoflagellates was acquired by a single secondary endosymbiosis in their common ancestor (Archibald 2005, Archibald 2008). The process of serial endosymbiotic events explains the diversity of photosynthetic eukaryotes and is responsible for the occurrence of photosynthesis throughout the eukaryotic tree of life.

The *green algae* are photosynthetic eukaryotes characterized by the presence of chloroplasts with two envelope membranes, stacked thylakoids, the chlorophylls *a* and *b* and accessory pigments such as beta carotene and xanthophylls. They produce starch as the main storage product, which is deposited inside the plastids. Few genera like *Prototheca, Polytoma, Polytomella,* and *Hyalogonium* are not pigmented, but the cells contain leucoplasts, which secondarily lost their pigments (Pringsheim 1963). Many green algae live in symbioses with fungi (building lichen), protozoa and foraminifers, or even as parasites on tropical plants (Pröschold and Leliaert 2007). There are estimated to be at least 600 genera with 10,000 species within the green algae (Norton et al. 1996).

Modern classifications based on molecular data sets led to the description of two major lineages within the green algae (Fig. 1.1). The *Chlorophyta* are commonly called green algae while the other lineage is named Streptophyta that includes charophyte algae and embryophyte land plants (Lewis and McCourt 2004).

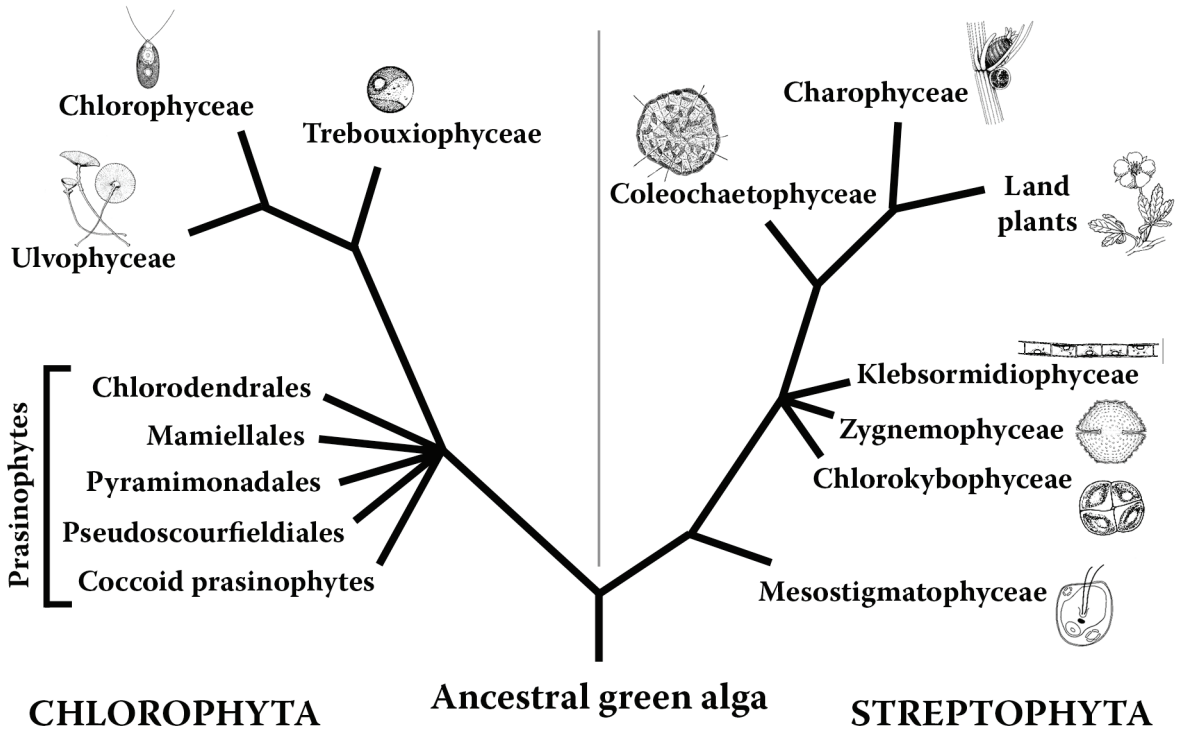

Fig. 1.1: Summary of the phylogenetic relationships among the major lineages of green algae determined by analysis of DNA sequence data (Rindi 2010, modified after Lewis and McCourt 2004).

It has been shown that transitions from aquatic to *terrestrial* habitats occurred several times independently (Lewis and Lewis 2005, López-Bautista et al. 2007, Cardon et al. 2008). Thus, terrestrial green algae are a highly polyphyletic group originating from the classes Ulvophyceae, Chlorophyceae and Trebouxiophyceae (Chlorophyta) as well as Klebsormidiophyceae, Zygnemophyceae and Chlorokybophyceae (Streptophyta). The here investigated green algal biofilms are dominated by *Trebouxiophyceae.* They are characterized by a combination of ultrastructural characteristics: counterclockwise orientation of the basal bodies, non-persistent metacentric mitotic spindles and the presence of a phycoplast, none of which is unique to the class. The basal body orientation is shared with the Ulvophyceae, metacentric spindles with the prasinophytes and nonpersistent spindles and phycoplasts with the Chlorophyceae (Cocquyt 2009). The Trebouxiophyceae were described as a monophyletic class on the basis of 18S rRNA sequence data (Friedl 1995). However, more recent molecular studies do not recover this suggested monophyly or recovered only weak support for monophyly (Krienitz et al. 2003, Pröschold and Leliaert 2007, Darienko et al. 2010), hence this question will need further attention. Trebouxiophyceae occur as coccoidal or ellipsoidal unicells (Fig 1.2 a, b), sarcinoid colonies (Fig 1.2 c), short filaments or small blades (e.g. *Prasiola*).

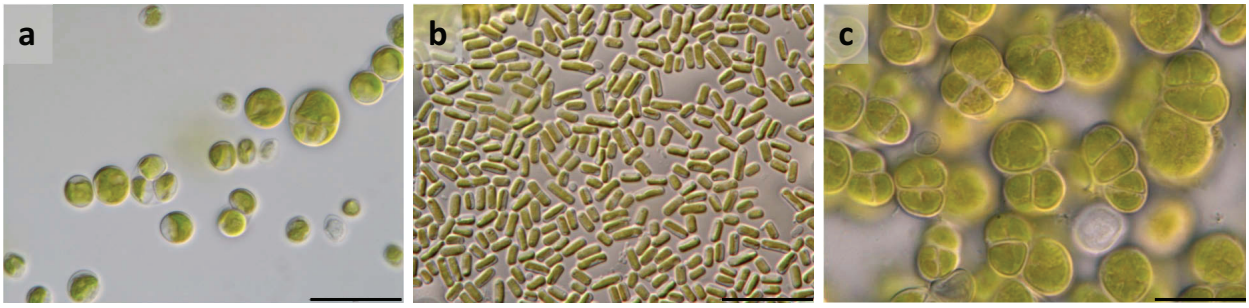

Fig. 1.2: Typical morphotypes in trebouxiophyceaen green algae. **a** *Jaagichlorella* sp. SAG 2196, **b** *Stichococcus bacillaris* SAG 379-1b and **c** *Apatococcus lobatus* SAG 2096. Pictures a, b in courtesy of T. Darienko, Kiew, c by O. Mudimu, Kiel.. *Scale bar* 20 µm

Ecophysiology

The term ecophysiology combines the disciplines ecology and physiology. Consequently, it studies the adaptation of (algal) physiology to environmental conditions. The mechanisms controlling growth, reproduction, survival, abundance, and biogeography of any organism is affected by physical, chemical and biotic parameters. The knowledge of adaptation strategies (morphological, physiological and biochemical) of aeroterrestrial green algae to their challenging environment leads to a deeper understanding of functional significance of specific algal traits and their evolutionary performance during the colonization of terrestrial habitats.

2.1 Aims

This thesis resulted from a DFG project entitled “Green algae-dominated biofilms on artificial hard substrates: phylogenetic-taxonomical analysis of species composition and ecophysiological characterization of algal assemblages”. Natural biofilms and unialgal isolates of aeroterrestrial green algae (Trebouxiophyceae) have been investigated by an interdisciplinary approach combining molecular taxonomy with ecophysiology and biochemistry. The present work focuses on identification of tolerance limits, adaptation strategies and the role of certain biomarkers in chemotaxonomy. Following this scope, I aimed to answer the following questions:

- Which chemical or physiological characters are useful for green algal systematics on the family level within the class Trebouxiophyceae?
- What are the key factors for algal growth in aeroterrestrial biofilms?
- Which are the major adaptation strategies of aeroterrestrial green algae to the harsh environmental conditions in the terrestrial habitat?

Profound knowledge of ecophysiological traits is the key factor to understand algal distribution and competitive relationships, which finally contributes to a sustainable prevention of biofouling.

2.2 Outline

The results **(Chapter 3)** are presented in terms of three publications, one submitted manuscript and one manuscript in preparation.

Chapter 3.1 presents a polyphasic approach investigating morphological, molecular, biochemical and physiological characters of ellipsoidal *Chlorella*-like algae (Darienko et al. 2010), resulting in their taxonomical transfer to the genus *Chloroidium* NADSON. These species occur in all kinds of habitats and represent a significant component of aeroterrestrial green biofilms (Mudimu 2008). Extensive analysis for carbohydrate composition in *Chloroidium* spp. revealed the presence of the polyol ribitol as a characteristic protective compound. Further, investigation of growth in dependence of temperature indicated similar growth optima and rates in all studied *Chloroidium* species, which were significantly different from those of *Chlorella vulgaris*.

Chapter 3.2 summarizes results of an extensive polyol screening within the Trebouxiophyceae (Gustavs and Karsten, submitted). The analysis of polyol pattern allows an accurate discrimination of morphological similar and hence difficult to distinguish taxa such as *Apatococcus* and *Desmococcus*, and provides an economical analytic method for species identification on different taxonomical levels (compared to molecular methods).

Chapter 3.3 provides a methodological discussion about accuracy and limits of microalgal growth rate measurements by *in vivo* growth fluorometry (Gustavs et al. 2009a). From an ecological perspective, growth rate represents the most relevant process to describe the physiological performance of species because it integrates all intracellular (positive and negative) metabolic processes. Thus, a fast and simple evaluation of tolerance limits, growth optima and acclimation potential may facilitate the interpretation of natural distribution.

Chapter 3.4 represents a study investigating osmotic and matric stress in green algae from terrestrial, marine and freshwater habitats (Gustavs et al. 2009b). The comparison between growth responses under liquid and on solid substrate conditions (100% air humidity) revealed wide tolerance limits of aeroterrestrial green algae towards diminished water availability. Using ^{13}C nuclear magnetic resonance (NMR) and High Performance Liquid Chromatography (HPLC) the polyol ribitol was verified in free-living aeroterrestrial green algae, acting as a compatible solute.

Chapter 3.5 investigates the role of mixotrophy in metabolic performance of biofilm-dominating *Apatococcus lobatus* and discusses the relevance of mixotrophy in terrestrial biofilms (Gustavs et al. in prep). Mixotrophy is a well known phenomenon in nutrient- and light energy-poor systems with high organic substrate supply, enabling "primary producers" to function at multiple trophic levels. Terrestrial biofilms exhibit similar characteristics: intermediate to deeper layers are light-limited and probably well supplied with organic substrates. To evaluate the significance of mixotrophy for competitive success of *A. lobatus*, growth performance, photosynthetic activity and light acclimation were analysed comparing autotrophic with mixotrophic conditions. Using microsensors light climate was measured for the first time in natural aeroterrestrial algal biofilms and pure-culture colonies of *A. lobatus* to characterize vertical attenuation and spectral composition of radiation.

Finally, the main conclusions resulting from the biodiversity and ecophysiology investigations are summarized and discussed in **Chapter 4** and perspectives for future research are provided.

Original Paper

CHLOROIDIUM, A COMMON AEROPHYTIC COCCOID GREEN ALGA PREVIOUSLY ASSIGNED TO CHLORELLA (TREBOUXIOPHYCEAE)

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Research Note

POLYOLS AS CHEMOTAXONOMIC MARKERS TO DIFFERENTIATE BETWEEN AEROTERRESTRIAL GREEN ALGAE (TREBOUXIOPHYCEAE, CHLOROPHYTA)[1]

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Abstract

Neither the conventional morphological nor the modern molecular species concept result in a satisfying taxonomical system for microalgae. This is especial true for aeroterrestrial members of the Trebouxiophyceae, which often exhibit simple but ambiguous morphotypes. Therefore, polyphasic approaches combining molecular, morphological or physiological traits with chemical characters can lead to an improved designation of certain strains to different classes or orders. Polyols are known as important organic osmolytes and compatible solutes in several algal lineages, and additionally have been successfully used as chemotaxonomical markers in ancestral red algae.

In this study, the distribution of polyols was examined in 35 green algal strains from 5 different clades belonging to the classes Trebouxiophyceae and Chlorophyceae. Sorbitol was detected in representatives of the *Prasiola*-clade, while ribitol was present in the *Elliptochloris*- and *Watanabea*-clade. *Apatococcus lobatus*, as member of the *Watanabea*-clade, occupied an exceptional position as it contained erythritol in addition to ribitol. Members of the order Chlorellales as well as two representatives of the class Chlorophyceae did not contain polyols. Thus, the constitutive presence of specific polyols facilitates a differentiation between species of the *Prasiola*-clade from the *Elliptochloris*- and *Watanabea*-clade, respectively. The existence of erythritol in *A. lobatus* might even permits

identification on the genus level. The data indicate a high chemotaxonomical value for polyols in trebouxiophyceaen taxonomy.

Keywords: aeroterrestrial green algae · biofilms · chemotaxonomy · polyols · Trebouxiophyceae

The identification of green microalgae has been traditionally based on microscopical observations of natural samples and unialgal cultures (Ettl and Gärtner 1995, Graham and Wilcox 2000). Usually, their representatives are 5- 15 µm in diameter and exhibit simple morphotypes as single cells, sarcinoid cell packages or unbranched filaments (Rindi 2007). Under natural conditions, aeroterrestrial green algae build up thick cell walls and mucoid layers, complicating morphological identification due to uniform appearance (Karsten et al. 2005 a, 2007 a). Furthermore, a high phenotypic plasticity has been demonstrated for these algae in several studies (Rindi and Guiry 2002, Luo et al. 2006, Darienko et al. 2010), leading to additional taxonomical uncertainties. Despite of simple and uniform morphology within the aeroterrestrial algae, molecular investigations based on rDNA sequences revealed a surprisingly high diversity at the species level (Lewis and Lewis 2005, Lopez-Bautista et al. 2007, Mudimu 2008, Rindi 2010). Nevertheless, molecular data sets such as clone libraries do not reflect the quantitative appearance of certain taxa, they rather provide presence-absence data (Luo et al. 2010). The recent situation for green microalgae is characterized by the quest for a compromise between the conventional (morphological) and the modern phylogenetical system (Coesel and Krienitz 2008). A combination of several disciplines in polyphasic approaches seems promising for identification and delimitation of species and genera (Pröschold and Leliaert 2007, Coesel and Krienitz 2008, Darienko et al. 2010).

The concept of chemotaxonomy is based on the assumption, that genotypic differences are also reflected by the presence of chemical characters. An organic compound is suitable as chemotaxonomical marker, if it is specific to a certain taxa or group of organism, sufficiently abundant to detect and consistent within a lineage (Karsten et al. 2007b). The occurrence or lack of specific carbohydrate components, such as major storage compounds, cell-wall constituents or low molecular weight photosynthates, can be used for algal systematics (Darienko et al. 2010 and references therein). Low molecular weight carbohydrates (LMWCs) as polyols and heterosides have been successfully used for chemotaxonomical differentiation within the ancestral red algae (for review see Eggert and Karsten 2010). In addition, polyols are synthesized by several green algal taxa (lichen photobionts: Honegger et al. 1967, Lewis and Smith 1967, *Trentepohlia* sp.: Feige and Kremer 1980, *Prasiola crispa*: Jacob et al. 1991, aeroterrestrial green algae: Gustavs et al. 2009). In the present study, we screened isolates of

abundant aeroterrestrial green algae (Trebouxiophyceae) for their polyol pattern in comparison to morphological similar representatives of the Chlorophyceae. The results were related to the existing taxonomic system for green algae (Lewis and McCourt 2004, Pröschold and Leliaert 2007) to assess the applicability of polyols as chemotaxonomic markers in order to provide a fast and efficient method for at least higher rank differentiation between morphological similar green algae.

A total of 35 green algae (33 unialgal cultures from the Sammlung of Algenkulturen at the University of Göttingen (SAG, Göttingen, Germany), 2 field samples) belonging to 10 different clades were investigated (Tab. 1). The cultures were grown at 20-22°C under constant irradiation (Osram Lumilux Deluxe Daylight, 40 µmol photons · m^{-2} · s^{-1}, 16:8 light:dark cycle) using modified Bolds Basal Medium (MBBM) (Starr and Zeikus 1993). After incubation for 20-30 days, cells were harvested by centrifugation for 5 min at 6240 x g. The algal pellets were lyophilized (20 h, Lyovac GT2, Seris GmbH, Hürth, Germany) and stored dry and dark at room temperature. Dry algal samples of 7-12 mg dry weight were extracted with 1mL 70% aqueous ethanol (*v/v*) in capped centrifuge tubes at 70°C for 4 h according to Karsten et al. (1991). After centrifugation for 5 min at 5000 x g, 0.7 mL of the supernatant was evaporated to dryness under vacuum (Savant SpeedVac SPD111). Dried extracts were re-dissolved in 0.7 mL distilled water and vortexed for 30s. Samples were analysed with an isocratic Agilent HPLC system equipped with a differential refractive index detector. LMWCs were separated and quantified by two established HPLC methods (Karsten et al. 1991, Karsten et al. 2005b) in order to maximize peak identification. Separation of polyols, mono- and disaccharides was performed on a Bio Rad resin-based column (Aminex Fast Carbohydrate Analysis, 100 x 7.8 mm) using a Phenomenex Carbo-Pb^{2+} (4 x 3 mm) guard cartridge. LMWCs were eluted with 100% HPLC grade water at the flow rate of 1 mL min^{-1} at 70°C (modified after Karsten et al. 1991). Separation of heterosides and polyols was performed on a Phenomenex resin-based column Rezex ROA-Organic Acid (300 x 7.8 mm) protected with a Phenomenex Carbo-H^+ guard cartridge (4 x 3 mm). LMWCs were eluted with 5 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1} at 75°C (modified after Karsten et al. 2005b). LMWCs were identified by comparison of retention times with those of standard compounds ribitol, sorbitol and erythritol (Sigma-Aldrich, St. Louis, USA) prepared as 1 mM aqueous solutions and quantified by peak areas. The concentrations are expressed in µmol g^{-1} dry mass. The presence of ribitol and erythritol was identified by ^{13}C NMR (nuclear magnetic resonance) analysis. For NMR spectroscopy, an ethanolic extract of 100 mg dry mass of *Apatococcus lobatus* (Chodat) Boye Petersen (SAG 2096) was prepared as for HPLC analysis, but was redissolved in 0.5 ml D_2O. The ^{13}C NMR spectra were recorded with a Bruker AVANCE 500 spectrometer operating at 125.8 MHz for ^{13}C. A sweep width of 30,000 Hz, 32,000 time domain points, and a 30° pulse of 3.0 µs were used for acquisition, with a composite pulse decoupling (number of

scans: 20,000). Samples were run at a temperature of 27°C and were referenced to the resonance of external acetone (δ = 31.1 ppm). A reference spectrum of pure erythritol has been recorded under the same conditions and proved to be identical with the spectrum recorded for the SAG 2096 extract.

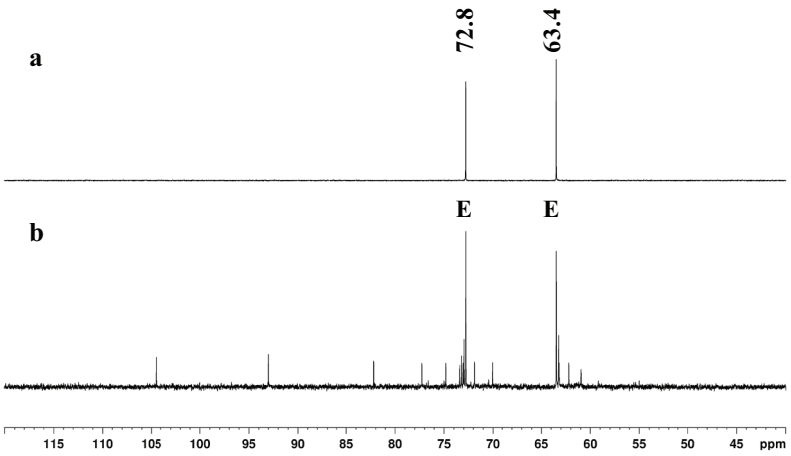

Fig 1: **a** ^{13}C NMR of pure erythritol and **b** of the ethanolic extract of *Apatococcus lobatus* SAG 2096 in D_2O. Resonances at 72.8 and 63.4 ppm relate to carbon atoms in erythritol. **E** signals of erythritol.

The presence of ribitol in various aeroterrestrial green algae has been reported earlier (as well proven by HPLC and ^{13}C NMR analysis, Gustavs et al. 2009). While erythritol exhibited diagnostic ^{13}C resonances at 63.4 and 72.8 ppm (Fig. 1), those of ribitol were at 62.4, 72.1 and 72.2 ppm (Gustavs et al. 2009).

The osmotic function of polyols in eukaryotic algae has been investigated extensively (for review see: Oren 2007, Eggert and Karsten 2010) while their chemotaxonomical value has only been assessed for ancestral red algae so far (Karsten et al. 2003). The green algal strains studied were selected due to their abundant occurrence in aeroterrestrial habitats (Jacob et al. 1991, Rindi and Guiry 2004, Mudimu 2008). Mudimu (2008) investigated 79 aeroterrestrial green biofilm samples applying molecular methods (gene cloning, sequencing, DGGE fingerprint analysis), identifying *Apatococcus* (75%) and *Chloroidium* (61%) as major components. In addition, *"Chlorella" luteoviridis* (27%), *Stichococcus* (17%) and *Coccomyxa* (11%) occurred frequently. All investigated algae from the *Watanabea*-, *Elliptochloris*- and *Prasiola*-clade contained polyols in significant concentrations (Tab. 1), indicating a high protective function for aeroterrestrial microorganisms (Karsten et al. 2007 a). It is assumed that the occurrence of polyols generally reflects harsh environmental conditions for the respective organism (Darienko et al. 2010). Sorbitol is specific to the *Prasiola*-clade, while ribitol occurs in the *Elliptochloris*- and *Watanabea*-

clade. Within the latter clade, *Apatococcus* revealed an exceptional position as it synthesizes erythritol in addition to ribitol (Tab. 1, Fig. 1). Erythritol has so far only been reported for *Trentepohlia* sp. (Ulvophyceae) (Feige and Kremer 1980). The investigated algae belonging to the *Chlorella*-clade as well as the Chlorophyceae lack polyols. Traditional taxonomical approaches often depend on single or even negative "characters" such as the absence of zoospore formation (Pröschold and Leliaert 2007). A chemical trait can be "absent" due to several reasons: i) its concentration is below the detection limit, ii) it is not constitutive and might be triggered by a certain environmental signal, iii) the organism is genetically not able to express it. Due to those uncertainties, verification of positive characters is preferable for classification instead of the absence of a distinct trait. Chemotaxonomical identification, for example, of *Chloroidium* can be performed by the presence of ribitol, while *Chlorella* typically contains the rather unique chemical marker ergosterol (Görs et al. 2010 submitted). These authors investigated the distribution of sterols in Chlorellaceae and morphological similar species, detecting ergosterol exclusively in the *Chlorella*- and *Parachlorella*-clade, while *Chloroidium* or *Scenedesmus Scenedesmus* (Chlorophyceae) lack this cell wall constituent.

Tab. 1: Concentrations of polyols in the investigated Trebouxiophyceae. All cultures were harvested in the stationary phase of growth. Given are clade and species affiliation (according to Mudimu (2008) and Darienko et al. 2010), strain numbers (SAG –Culture Collection Göttingen), and concentrations of polyols as µmol g^{-1} dry weight; mean value ± SD, n=3 (* n=1); n.t.: no trace. [1]Darienko et al. 2010

Class	Clade	Species	SAG Number	concentration of polyols in µmol g^{-1} dw Ribitol	Erythritol	Sorbitol
Trebouxiophyceae	*Watanbea*- clade	*Apatococcus lobatus* (Chodat) Boye Petersen	2037	21.8 1.8	387.9 18.3	n.t.
		Apatococcus lobatus (Chodat) Boye Petersen	2145	66.5 1.5	359.9 18.8	n.t.
		Apatococcus lobatus (Chodat) Boye Petersen	2096	189.7 4.1	321.3 19.4	n.t.
		Apatococcus lobatus (Chodat) Boye Petersen	2199	121.1 16.8	375.0 34.9	n.t.
		Apatococcus lobatus (Chodat) Boye Petersen	2151	63.8 *	184.8 *	n.t.
		Apatococcus lobatus (Chodat) Boye Petersen	2072	222.4 13.1	304.4 27.6	n.t.
		Chloroidium ellipsoideum (Gerneck) Fott & Nováková	6 strains [1]	95 -446	n.t .	n.t.
		Chloroidium saccharophilum (Krüger) Migula	2149	72 *	n.t.	n.t.
		Chloroidium saccharophilum (Krüger) Migula	2197	61 *	n.t.	n.t.
		Chloroidium saccharophilum (Krüger) Migula	2120	70 *	n.t.	n.t.
		Chloroidium angusto -ellipsoideum (Krüger) Migula	2141	27 *	n.t.	n.t.
		Chloroidium angusto - ellipsoideum (Krüger) Migula	2041	91 *	n.t.	n.t.
		Chloroidium angusto - ellipsoideum (Krüger) Migula	2115	33 *	n.t.	n.t.
	Elliptochloris -clade	*Chlorella luteoviridis* Chodat	2196	360.3 23.2	n.t .	n.t .
		Chlorella luteoviridis Chodat	2213	249.8 5.5	n.t .	n.t .
		Coccomyxa viridis Chodat	216 -14	7.6 0.6	n.t .	n.t.
		Coccomyxa avemensis Jaag	216 -1	25.5 10.6	n.t.	n.t.
		Coccomyxa mucigena Jaag	216 -4	7.9 0.7	n.t.	n.t.
		Coccomyxa sp.	2040	47.4 3.0	n.t.	n.t.
	Prasiola -clade	*Prasiolopsis ramosa* Vischer	26.83	n.t.	n.t.	105.3 11.6
		Prasiola crispa (Lightfoot) Kützing	-	n.t.	n.t.	180.5 20.4
		Prasiola stipitata Suhr ex Jessen	-	n.t.	n.t.	179.1 14.0
		Stichococcus bacillaris Nägeli	379 -1b	n.t.	n.t.	237.2 12.8
		Stichococcus sp.	2060	n.t.	n.t.	144.7 8.5
		Stichococcus sp.	2059	n.t.	n.t.	99.4 6.4
		Stichococcus sp.	2119	n.t.	n.t.	128.2 2.6
		Desmococcus olivaceus (Persoon ex Acharius) Laundon	1.92	n.t.	n.t.	72.1 8.9
		Desmococcus olivaceus (Persoon ex Acharius) Laundon	1.94	n.t.	n.t.	221.0 18.7
		Desmococcus endolithicus Broady & Ingerfeld	25.92	n.t.	n.t.	47.2 *
	Chlorella- clade	*Chlorella vulgaris* Beijerinck	211-11 b[1]	n.t.	n.t.	n.t.
		Chlorella sorokiniana Shihira et Krauss	211-31	n.t.	n.t.	n.t.
		Chlorella lobophora Andreeva	37.88	n.t.	n.t.	n.t.
		Parac hlorella kessleri (Fott & Nováková) Krienitz et al. 2004	211 -11 a	n.t .	n.t .	n.t .
		Parachlorella beijerincki Krienitz et al. 2004	2046	n.t .	n.t .	n.t .
Chloro-phyceae		*Scenedesmus rubescens* (Dangeard) Kessler et al. 1997	5.95	n.t .	n.t .	n.t .
		Scenedesmus vacuolatus Shihira & Krauss	211 -8b	n.t .	n.t .	n.t .

The taxonomical value of polyol distribution pattern in morphologically very similar, but phylogenetically distinct *Apatococcus* and *Desmococcus* (Fig. 2) is high. Both genera are very abundant in aeroterrestrial biofilms, especially in urban areas (Rindi and Guiry 2004, Rindi 2007, Mudimu 2008), where they cause extensive discolorations on walls and roofs. Due to their similar microscopic appearance (Fig. 2), members of both groups were initially attributed to a proto-pleurococcoid "community" or *Pleurococcetum* (Rindi 2007 and references therein).

The occurrence of Prasiolales (including *Desmococcus*) is associated with more moist regions of Atlantic Europe (in particular Ireland and North-western Spain), while *Apatococcus* is dominant at moderate continental sites; nevertheless, also mixed communities have been observed (Rindi 2007). Extraction and analysis of polyols from natural biofilms in combination with microscopical observations will give fast and definite information about taxonomical composition of these assemblages, which to date is a matter of discussion (Rindi 2010).

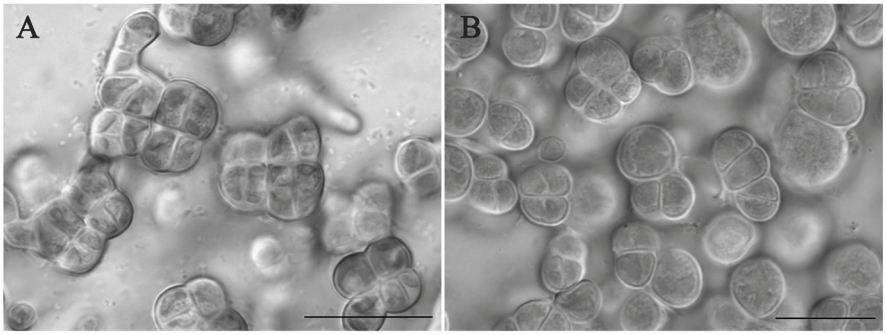

Fig. 2: Light microscopy of **A** *Desmococcus olivaceus* SAG 1.92, **B** *Apatococcus lobatus* SAG 2096, *Scale Bar:* 20 µm

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Original Paper

IN VIVO GROWTH FLUOROMETRY: ACCURACY AND LIMITS OF MICROALGAL GROWTH RATE MEASUREMENTS IN ECOPHYSIOLOGICAL INVESTIGATIONS

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Original Paper

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF GREEN MICROALGAE FROM DIFFERENT HABITATS TO OSMOTIC AND MATRIC STRESS

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THE ROLE OF MIXOTROPHY IN METABOLIC PERFORMANCE OF *APATOCOCCUS LOBATUS* (TREBOUXIOPHYCEAE, CHLOROPHYTA), AN ABUNDANT AEROTERRESTRIAL GREEN ALGA [1]

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Abstract

The green microalga *Apatococcus lobatus* colonizes aeroterrestrial habitats throughout many climatic zones. It is complicated to isolate, difficult to culture and slow growing under laboratory conditions. However, *A. lobatus* frequently dominates green biofilms on natural and artificial substrata. Biofilm formation is discussed as physical protection against harsh environmental conditions like excessive radiation or water deficiency. It is supposed that photo- and heterotrophic organisms support each other's lifestyle in these communities by exchange of CO_2 versus O_2 as well as degradation of

particulate organic carbon. The ability of *A. lobatus* to grow mixotrophically is therefore assumed as a competitive advantage in these organic rich habitats. Under mixotrophic conditions, pigment pools, photosynthetic rate and xanthophyll cycle are modified in *A. lobatus*. Growth rate remains equally low as under pure autotrophic conditions, while growth capacity is significantly enhanced due to high carbon concentrations in the organic medium. Microscale radiation measurements revealed that irradiance is strongly attenuated with increasing biofilm depth and serious self-shading occurs. Mixotrophy is regarded as a major competition strategy in such light-limited habitats, enabling primarily phototrophic organisms to meet their energy and carbon demand from organic substrates, making them less dependent on radiation supply. The ecological success of *A. lobatus* under natural conditions can be partly explained by its ability to combine different metabolic modes and withstand radiation deficiency. The mixotrophic culturing approach clearly enhances accumulation of biomass, thereby permitting the future application of *A. lobatus* as a model organism investigating biodeterioration processes at anthropogenic hardsubstrates as facades or roof tiles.

Key index words: abundance · aeroterrestrial biofilms · growth · light climate · metabolites · mixotrophy · photosynthetic rate

Introduction

Terrestrial algal biofilms colonize the interface between a wide range of different surfaces and the atmosphere. They consist of complex microbial biocoeneses hosting algae, bacteria, fungi and amoebae (Gaylarde and Morton 1999). Aeroterrestrial green algae grow on (and partly in) various natural surfaces such as tree barks, rocks and soils (for review see Karsten et al. 2007, Gorbushina 2007). In urban areas, extensive algal biofilm formation on facades, roof tiles and wooden surfaces constitutes a biofouling problem (e.g. Rindi 2007).

Green algae of the aeroterrestrial genus *Apatococcus* are considered to have a cosmopolitan distribution (Edlich 1936, Ettl and Gärtner 1995, Rindi 2007) and are abundant on various natural and artificial substrata. *Apatococcus* occurs epiphytically (Gärtner 1974, Søchting 1997), as soil algae (Gärtner and Ingolic 1989) and on anthropogenic hard substrates (e.g. Rindi and Guiry 2004, Uher et al. 2005, Karsten et al. 2007, Rindi 2007). Its occurrence has been reported from Europe (Darienko and Hoffmann 2003, Karsten et al. 2005, Flores et al. 1997, Søchting 1997), Asia (Handa and Nakano 1988) and Africa (Gubanski et al. 2000). Mudimu (2008) demonstrated an always high abundance of

Apatococcus spp. in 86 biofilms from central Europe (mostly Germany) using 18S rRNA gene sequencing. Although there exist some data about morphology, life-history and reproduction (Gärtner and Ingolic 1989) as well as about biochemical aspects such as lipid profiles (Zarnowski 2002, Eichel and Zarnowski 2001, Zarnowski et al. 2000), only little knowledge is available about the ecophysiological performance and acclimation strategy that enable *Apatococcus* spp. to dominate these terrestrial habitats (Edlich 1936, Bertsch 1966, Gustavs et al. 2009b). The conspicuous dominance of *Apatococcus* in many aeroterrestrial biofilm communities (in contrast to *Chloroidium*, *Stichococcus*, *Coccomyxa* and *Klebsormidium*) is still an unexplained phenomenon.

Generally, aeroterrestrial microalgae can be regarded as euryoecious concerning the tolerated amplitudes, strengths and combinations of environmental parameters (Gorbushina 2007, Karsten et al. 2007). Compared to aquatic algae, aeroterrestrial representatives are exposed to harsher environmental conditions, such as high diurnal and seasonal fluctuations in PAR and UVR, strong temperature fluctuations and restricted water availability (Karsten et al. 2007). Excessive radiation inhibits many biological processes resulting in photoinhibition or even photodamage which in turn leads to a decrease or inhibition in growth and reproduction (Franklin and Forster 1997). Biofilm formation is discussed as a strategy to counteract such harmful situations. Outer cells absorb most of the radiation energy and often bleach, thereby protecting inner cells effectively from photodamage (Franklin and Larkum 1997, Stal 1995). More recent data on green algae occurring in biotic desert crusts (North America) indicate that shading effects due to such three-dimensional spatial arrangements may be ecologically highly important (Gray et al. 2007). Despite their ability to withstand desiccation, these algae were surprisingly susceptible to photodamage at photon fluence rates of only 130 µmol m^{-2} s^{-1} which is low compared to full sunlight intensity experienced by these crusts. Gray et al. (2007) suggested that under *in situ* conditions these algae probably occupy microenvironments within the crust where they are protected from damaging light levels. Nevertheless, the spatial zonation of aeroterrestrial biofilms, e.g. occurrence of different algal species at certain positions according to their tolerance levels and competitive strength, has not been studied so far. Unlike filamentous cyanobacteria and benthic diatoms in aquatic microbial mats, aeroterrestrial green algae are immotile and, thus, unable to migrate to the most suitable layer inside the biofilm (Karsten et al. 2007). Besides physical protection through biofilm formation, physiological and metabolical mechanisms such as the xanthophyll cycle (e.g. Masojídek et al. 2004) and the synthesis and accumulation of UV-sunscreens (Reisser et al. 2001, Karsten et al. 2007) are expressed.

Most microorganisms can be divided into those relying solely on photosynthesis (phototrophy) or those depending exclusively on the assimilation of organic compounds (heterotrophy) to meet their

requirements for energy and carbon supply. In contrast, mixotrophic organisms can combine both nutritional modes, depending on substrate availability and abiotic conditions (Sanders et al. 1990, Cloern and Dufford 2005). Mixotrophy was recognized as a widespread phenomenon among some flagellate groups (Granéli and Carlsson 1998) whereas the majority of phytoplankton organisms (as base of aquatic food webs) were traditionally regarded as strict phototrophs (e.g. Hutchinson 1961). However, Pringsheim (1963) considered heterotrophy as a frequent nutritional mode within all algal taxa and even questioned the existence of pure phototrophic organisms (e.g. regarding the supply with essential vitamins). Accordingly, Tuchman (1996) assumed that most algae are physiologically capable to metabolize in both nutritional pathways, and although autotrophy is employed most frequently by algae, obligate autotrophy is probably less common than was conventionally thought. To date, mixotrophy is discussed as typical nutritional mode in oligotrophic phytoplankton assemblages (Sanders 1991, Tittel et al. 2003) as well as in organic-rich and light energy-poor environments such as estuaries which often favour harmful algal blooms (Burkholder et al. 2008).

Atmospheric aerosols, gases, propagatory particles and rain serve as external sources of nutrients for aeroterrestrial algal assemblages (Gorbushina 2007, Karsten et al. 2007), especially near agricultural land-use. Internal nutrient sources for such biofilms are provided by photosynthetic exudates and/or degradation of extracellular polymers such as polysaccharides, while the remineralisation of dead algal cells by heterotrophic organisms could be considered as recycling of nutrients supporting new growth of phototrophic organisms (Karsten et al. 2007). Consequently, potential nutrient limitation can be at least partly counteracted by the expression of hydrolytic enzymes aiding remineralization in the mucoid matrix (Thompson and Sinsabaugh 2000). Hetero- and autotrophic organisms hosting biofilms are generally believed to support each other lifestyle, thus symbiotic and mutualistic interactions are likely (Yallop et al. 1994, Stal 1995, Gorbushina and Broughton 2009). The existence of *Apatococcus* in lichen symbiosis is a matter of debate (Beck and Persoh 2009) but, however, a close association with fungi is frequently observed (Petersen 1932b, Edlich 1936, Ettl and Gärtner 1995, Hallmann 2007, Freystein et al. 2008). The association of algae with aerobic bacteria has as well been frequently recorded. Bacteria decompose and utilize (dead) algal material and extracellular products (Bell 1983), providing in turn carbon dioxide and vitamins to the algae (Mouget et al. 1995). Zarnowski et al (2002) observed a close association between the terrestrial green alga *Apatococcus constipatus* and aerobic bacteria postulating a symbiotic relationship. Watanabe et al. (2005) discussed mutualism and commensalism for the interaction between *Chlorella sorokiniana* and bacterial and fungal symbionts under photoautotrophic conditions.

In this study, the light climate inside an aeroterrestrial green algal biofilm as well as inside *Apatococcus lobatus* colonies on solid medium was measured using microelectrodes. The main goal was to describe the spatial zonation of vertical light penetration and availability to estimate the photosynthetic *in situ* activity. Further laboratory experiments were conducted with auto- and mixotrophically grown *A. lobatus* cultures. Photosynthetic performance was measured in differently adapted cultures under varying PFDs. Photoprotective mechanisms including xanthophyll cycle and regulation of pigment pools were evaluated under a wide range of radiation conditions. In addition, growth rates were determined under autotrophic and mixotrophic culture conditions. All data indicate that mixotrophy contributes to the ecological success of *Apatococcus lobatus.*

Material and Methods

Isolate and culture conditions. Unialgal cultures of aeroterrestrial *Apatococcus lobatus* SAG 2096 were autotrophically grown in modified Bolds Basal Medium (3N-BBM) (Starr & Zeikus 1993, BBM modified by addition of triple nitrate concentration) and in parallel mixotrophically by addition of 1 % glucose (w/v) to respective 3N-BBM. Stock cultures were kept at 20°C and 40 µmol photons m^{-2} s^{-1} under a light: dark cycle of 16: 8 h. Osram Daylight Lumilux Deluxe lamps were used as light sources. Radiation measurements were carried out with a Li-Cor LI-190-SB cosine corrected sensor connected to a Li-Cor LI-1000 datalogger (Lambda Instruments, Lincoln, USA, sensor error < 2%). Additionally, *A. lobatus* was grown under low and high light conditions, while all other parameters were as described above. Low light conditions (20 µmol photons m^{-2} s^{-1}) were achieved by covering the cultures with several layers of black gauze while enhanced radiation (100 and 500 µmol photons m^{-2} s^{-1}) was achieved by cultivation in a temperature-controlled water bath (± 0.1 K) (DC10 and K10; Thermo Haake) surrounded by light panels. All light incubations were performed under autotrophic and mixotrophic growth conditions. Bacterial abundance was regularly checked at both culturing modes and remained constantly low with about 0.05 x 10^6 cells ml^{-1} (data not shown).

Light climate in natural biofilms and colonies. The light climate measurements were performed on stationary phase *Apatococcus* colonies grown on agarose (culture age up to 4 month), which typically build three-dimensional assemblages up to 2 mm height. Bark samples (size approximately 2 cm^2) colonized by a natural biofilm were collected from the "weather"-side (north-orientation) of a *Tilia platyphyllos* tree in Rostock, Germany. The algal composition was microscopically checked at 40-fold magnification (Olympus BX 51 microscope). High spatial resolution measurements of scalar irradiance were performed with a fiber-optic scalar irradiance microprobe (integrating sphere diameter 10 µm, Lassen et al. 1992a) connected to a spectrometer (USB 4000, Ocean Optics, Dunedin, FL, USA), respectively. The down-welling spectral quantum irradiance was calculated according to Al-Najjar et al. (2009).

Growth performance. Growth rates were measured using an *in vivo* growth fluorometer (Hansatech MFMS, Norfolk, UK) according to the technique of Gustavs et al. (2009a), which monitors increases of *in vivo* chlorophyll *a* fluorescence F_t over time as an indicator of biomass accumulation (Karsten et al. 1996). Algae were cultured on 4 mL solid autotrophic and mixotrophic medium, respectively (3N-MBBM (+ 1 Glucose) + 1.5% Agarose) in disposable petri dishes with cover lids (Licefa, Bad Salzuflen, Germany) according to Gustavs et al. (2009b). Petri dishes were incubated under standard culture

conditions (see above) with 50 µl of log-phase cultures, which were evenly distributed on the agarose surface. Fluorescence measurements were performed in six replicates every 24 hours for up to 10 d. The standard error of 6 replicates was 14%. An apparent accumulation of biomass was observed after long-time cultivation for 4-6 months in mixotrophic cultures, which build large three-dimensional colonies on agarose medium. In contrast, SAG 2096 built only a thin cell-cover under autotrophic conditions, although the inoculum was equal for both conditions. The growth capacity for mixotrophic conditions was measured by weighting algal colonies (fresh biomass). The standard error of 3 replicates was 6%.

Pigment pools. Mixo- and autotrophically grown *A. lobatus* SAG 2096 was cultured for 3 weeks at 20, 40 and 500 µmol photons m^{-2} s^{-1}. Algal biomass was harvested by centrifugation (5000 x g, 2 min) in pre-weighted centrifuge tubes, frozen in liquid nitrogen, freeze-dried (Lyovac GT2, Seris GmbH, Hürth, Germany) and stored under dark, dry and cool conditions prior analysis.

Xanthophyll cycle. Exponentially growing cultures of mixo- and autotrophically cultured *A. lobatus* SAG 2096 were sampled at regular intervals over 24 h after an increase in photon flux density from 40 to 500 µmol m^{-2} s^{-1} at 20°C. Algal biomass was harvested by centrifugation (5000 x g, 2 min), frozen in liquid nitrogen, freeze-dried (Lyovac GT2, Seris GmbH, Hürth, Germany) and stored under dark, dry and cool conditions prior analysis.

Analysis of pigments. Pigments were extracted by adding 1 mL cold methanol/ ammonium acetate/ ethyl acetate solution (8:1:1). To break-up cells and maximize extraction yield, the mixture was homogenized under cool conditions by 3 x 10 s bead beating with 48.000 oscillations per min (Mini-BeadBeater, BioSpec Products, Inc., Bartlesville, OK, USA) under addition of several mg of glass beads (Sigma-Aldrich). Samples were centrifuged (4°C, 3 min, 6240 x g) to remove cell debris and glass beads. 500 µL of the clear supernatant were transferred into HPLC vials and mixed with 50 µL distilled H_2O in order to improve detection of slightly polar xanthophylls. Pigments were analysed on a SphereClone 5µ ODS(2) column (250 x 4.6 mm) (Phenomenex, Torrance, USA) protected with a C18 (4 x 3 mm) guard cartridge according to Ursi et al. (2003). Pigments were identified by spectra and retention time and quantified by co-chromatography with pure standards of chlorophyll *a*, chlorophyll *b*, neoxanthin, antheraxanthin, violaxanthin, zeaxanthin and lutein (DHI, Hoersholm, Denmark), concentrations are expressed in mg g^{-1} dw. The standard error of triplicates was 4- 23%. The quantification of xanthophylls within the high-light experiment was performed on the basis of Chl *a* concentration and is expressed in mmol mol^{-1} Chl *a*. The standard error of triplicates was 3- 19%.

Photosynthetic performance. Photosynthesis-irradiance (PI) curves (as µmol O_2 h^{-1} mg Chl a^{-1} vs PFD) were measured with an Oxygen Sensor Spot (SP-PSUP-YOP-D5) equipped with an fiber-optic oxygen meter Fibox 3 and the OxyView Software (PreSens GmbH, Regensburg, Germany, compare to Warkentin et al. 2007). Suspended algae were exposed to nine different PFDs up to about 500 µmol photons m^{-2} s^{-1}. Algae were grown at 20 and 100 µmol photons m^{-2} s^{-1}. PI curves were measured in triplicates and fitted to the equation of Walsby et al. (1997) when photoinhibition was evident

$$P = P_{\max\, pot} \cdot \left(1 - e^{\frac{-\alpha \cdot PFD}{P_{\max\, pot}}} \right) + \beta \cdot PFD + R$$

and to the equation of Webb et al. (1974) when photosynthesis was not inhibited at high PFDs

$$P = P_{\max} \cdot \left(1 - e^{\frac{-\alpha \cdot PFD}{P_{\max}}} \right) + R$$

with P = photosynthetic rate, P_{maxpot} = maximal photosynthetic rate without photoinhibition, α = slope of initial curve shape, PFD = photon flux density, β = slope under photoinhibition, R = respiration rate, P_{max} = maximal photosynthetic rate. The light saturation point of the PI curve I_k was calculated by dividing P_{max} by the initial slope α (Henley 1993).

Results

Light profiles in biofilms and colonies. Fig. 1A shows the spectral measurements of scalar irradiance inside a green algal colony of *Apatococcus lobatus* SAG 2096 with a diameter of about 450- 500 µm. The main absorption proceeds at 680 nm which belongs to the absorption maximum of Chl *a*. This wavelength is attenuated continuously with increasing depth between 50 and 250 µm, while below the 300 µm level it is quenched completely. Wavelengths around 560 nm and above 720 nm trespass through the whole colony but are attenuated strongly with a ratio of 70-90% at maximal depth. The spectral irradiance intensity in dependence of depth at wavelengths with maximal (680 nm) and minimal (560 and 800 nm) absorption by the algal cells is shown in fig. 1B. Irradiance of 680 nm attenuates by a factor of 10 every 100 µm, while 560 nm and 800 nm attenuate by the same factor every 340 and 640 µm, respectively. Fig. 1C illustrates the light attenuation at full PAR spectrum in a natural algal biofilm dominated by *A. lobatus*. The biofilm is about 200 µm thick and the wavelength of 680 nm is attenuated by a factor of 10 every 85 µm, while full PAR and 800 nm attenuate by the same factor every ~90 and 120 µm, respectively.

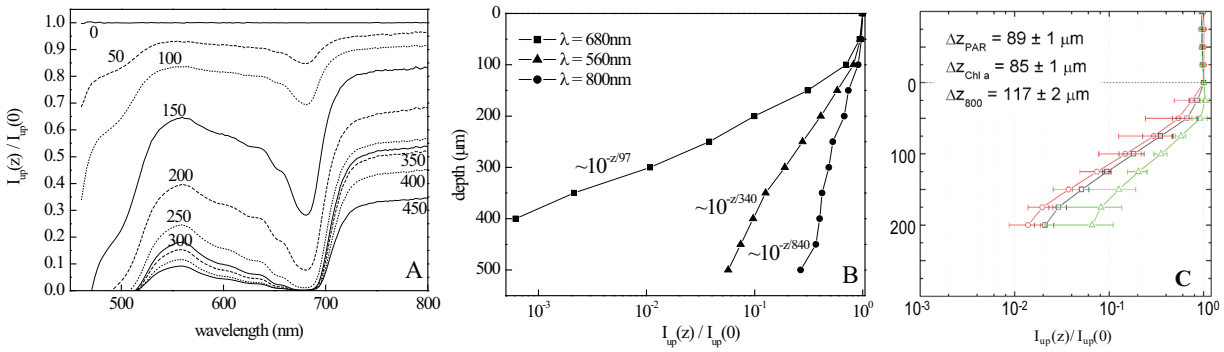

Figure 1: High spatial resolution measurements of scalar irradiance inside an algal colony (SAG 2096) **(A)** Spectral scalar irradiance (% of incident downwelling irradiance) measured at different depths (0- 450 µm in 50 µm steps). **(B)** Vertical microprofiles of light attenuation of different wavelengths. **(C)** Vertical microprofile of light attenuation in a natural aeroterrestrial biofilm (dominated by *A. lobatus*). (n = 3)

Growth performance. *Apatococcus lobatus* SAG 2096 grew under autotrophic culture conditions with a growth rate of 0.25 ± 0.03 d^{-1}, while under mixotrophic conditions growth rate accounted for 0.31 ± 0.04

d^{-1}. Although the growth rate in mixotrophic medium was slightly higher, no significant difference between both conditions was apparent (data not shown). The capacity for biomass accumulation was significantly enhanced under mixotrophic conditions. Colonies grown at glucose-enriched medium accounted for 61.6 ± 3.6 mg fresh mass, while biomass accumulation under autotrophic conditions reached only ~ 0.8 mg.

Photosynthetic performance. PI curves were measured for cultures grown at low (20 µmol) and intermediate (100 µmol) light conditions for both metabolical approaches. All approaches showed a pronounced low-light adaptation with light compensation points between 30 and 100 µmol photons $m^{-2}s^{-1}$. Under low light conditions, the maximal photosynthetic rate of the mixotrophic culture reached 12-18 µmol O_2 h^{-1} mg Chl a^{-1}, while the autotrophic culture exhibited a three times higher rate (fig. 3A). In contrast, autotrophic cultures grown under intermediate light conditions display a strong photoinhibition at PFDs above 100 µmol photons m^{-2} s^{-1} and in general a lower maximal photosynthetic rate than mixotrophic grown algae. Under intermediate light conditions, the mixotrophic algae displayed a higher photosynthetic rate, while the autotrophically grown culture exhibited a strong photoinhibition within low PFDs (Fig. 2B).

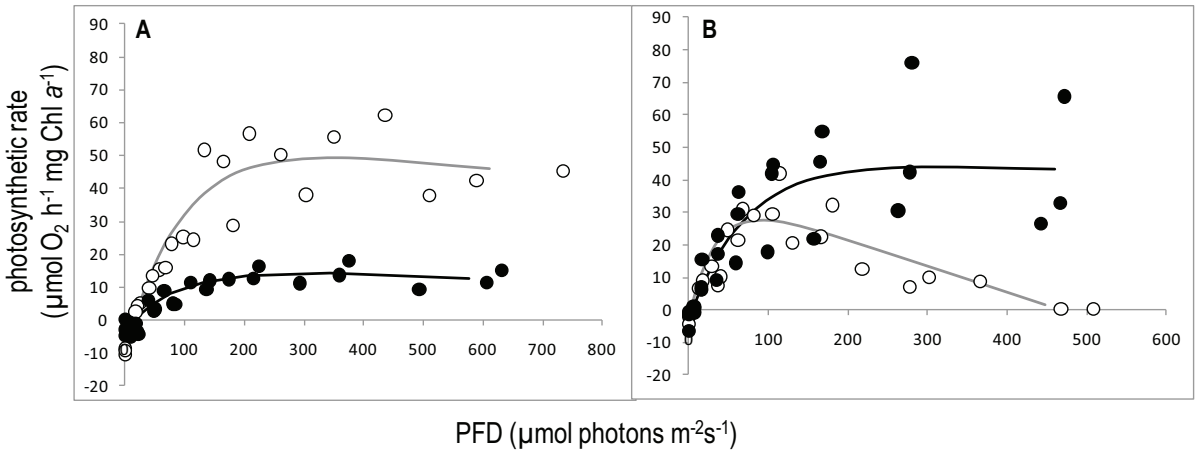

Figure 2: Photosynthetic rate (µmol O_2 h^{-1} mg Chl a^{-1}) of ●mixotrophic and ○autotrophic cultured *A. lobatus* cultivated at **A** 20 µmol photons $m^{-2}s^{-1}$ and **B** 100 µmol photons $m^{-2}s^{-1}$. (n = 3)

Pigment composition. The Chl *a* concentration in *A. lobatus* SAG 2096 varied significantly in stationary-phase cultures grown under different photon fluence densities. At intermediate PFDs (40 µmol photons m^{-2} s^{-1}) mixotrophic algae exhibited a Chl *a* concentration of 6.4 mg g^{-1} dry mass, about 6 times higher than in the autotrophic culture which showed 1 mg g^{-1} dry mass. Changing the light conditions from 40 µmol photons m^{-2} s^{-1} to 20 (low radiation) or 500 µmol photons m^{-2} s^{-1} (high radiation) resulted in strong differences in chl *a* concentrations of both nutritional modes (Fig. 3). While the autotrophic culture displayed a 5-fold up-regulation of Chl *a* concentration under the lowest light conditions, Chl *a* content in

mixotrophically cultured algae was almost identical at 20 and 50 µmol photons m^{-2} s^{-1}. Under high light conditions, algae from both nutritional modes showed comparable low concentrations of Chl *a* ranging from 1 to 2 mg g^{-1} dry mass. The lutein pool showed as well a pronounced enhancement at mixotrophic conditions and 40 µmol photons m^{-2} s^{-1}. At low light conditions, the differences in pool size disappear, while under high light conditions they are still evident but less pronounced (Fig. 3B). Although *A. lobatus* SAG 2096 under both culture conditions are able to regulate their Chl *a* and lutein concentrations in relation to external PFD, the light thresholds are different, i.e. they are located between 20 and 40 µmol photons m^{-2} s^{-1} for autotrophic cells, and between 40 and 500 µmol photons m^{-2} s^{-1} for mixotrophic cells (Fig. 3).

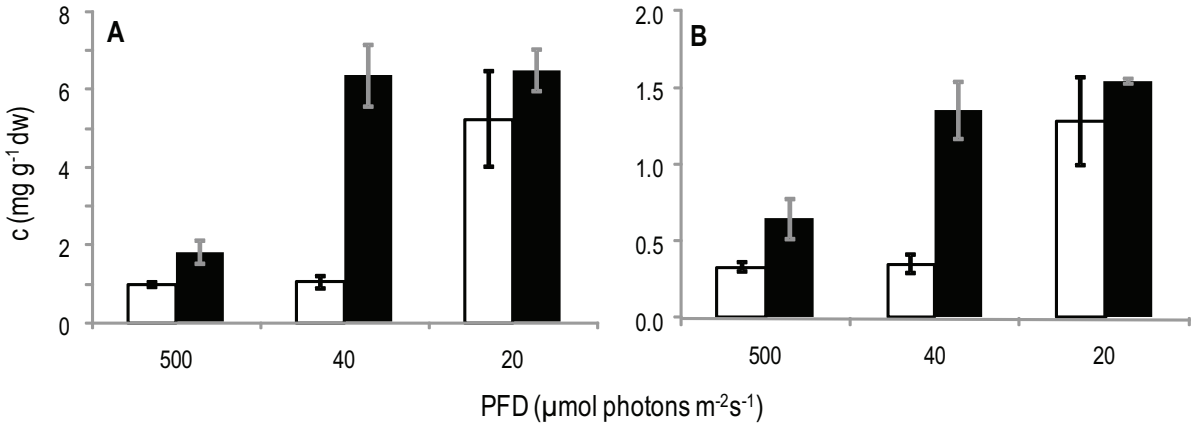

Figure 3: **A** Chl *a* and **B** lutein concentration (mg g^{-1} dw) in □ autotrophic and ■ mixotrophic cultures of *A. lobatus* under different photon flux densities. (n = 3)

Figure 4 illustrates the short-term regulation of xanthophyll concentrations (mmol mol^{-1} Chl *a*) in *A. lobatus* SAG 2096 after an abrupt increase of PFD from 40 to 500 µmol photons m^{-2} s^{-1}, comparing autotrophic with mixotrophic metabolism. Autotrophic cultures showed a complete conversion of violaxanthin to zeaxanthin with an initial violaxanthin concentration of 450 mmol mol^{-1} Chl *a* and a final zeaxanthin content of 470 mmol mol^{-1} Chl *a* (fig. 4A). In contrast, the mixotrophic algae contained already under steady state conditions an enhanced zeaxanthin concentration of 310 mmol mol^{-1} Chl *a* which was up-regulated to 815 mmol mol^{-1} Chl *a* during the high-light incubation period. Compared to the autotrophic cultures violaxanthin concentration was 2-fold lower at the beginning of the experiment, but down regulated to the same level during light treatment. Although a complete conversion of violaxanthin to zeaxanthin was evident in the mixotrophic approach it did not exclusively account for the strong increase in the observed zeaxanthin concentration (fig. 4B).

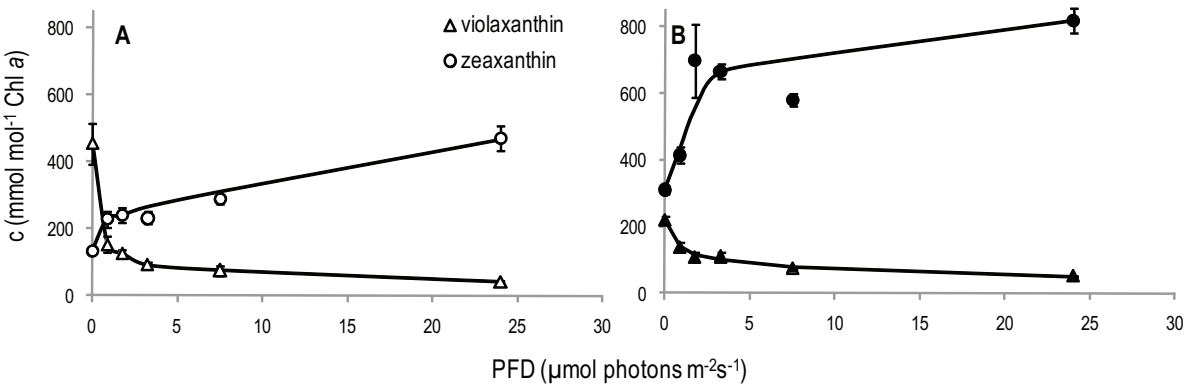

Figure 4: Conversion of viola- to zeaxanthin after a strong increase of radiation intensity from 40 to 500 µmol photons $m^{-2}s^{-1}$ in **A** autotrophic and **B** mixotrophic cultures of *A. lobatus*. (n = 3)

Discussion

Light climate in biofilms. While there are some field and laboratory studies evaluating light climate inside aquatic microbial mats which are usually dominated by cyanobacteria (Jørgensen et al. 1987, Jørgensen and des Marais 1988, Lassen et al. 1992, Kühl and Fenchel 2000, Al-Najjar et al. 2009) or in scleractinian corals (Ulstrup et al. 2006, Magnusson et al. 2007), this attempt has not been made for aeroterrestrial green biofilms. The natural biofilm investigated here was about 0.2 mm thick and dominated by *Apatococcus lobatus*, which forms dense, multi-layered biofilms on this substratum. The microscale measurements in respective biofilm demonstrate a strong exponential attenuation of scalar irradiance with depth, resulting in 1% of the surface level at 0.16 mm. The attenuation within the laboratory culture of *A. lobatus* is less pronounced and would reach 1% of the surface level at 0.7 mm, probably due to better water saturation (higher optical transparency) and a lower degree of aggregation (less self-shading). Directly below the surface (0- 0.05 mm), the scalar irradiance was slightly increased compared to the surface irradiance, due to multiple light scattering and photon trapping in the biofilm, as reported for microbial mat systems (Kühl and Jørgensen 1994, Kühl et al. 1996). The scalar irradiance inside a cyanobacterial mat from a tidal flat is attenuated exponentially with depth below 0.2 mm, resulting in 1% of the surface level at 1.2 mm depth (Al-Najjar et al. 2009).The light field within mats or biofilms is assumed to be highly heterogeneous due to variable size of components and interstitial rooms (Kühl and Jørgensen 1992, 1994, Kühl et al. 1994). In contrast to aeroterrestrial biofilms, aquatic microbial mats are water-saturated, mixed with sediment particles and further characterized by the spatial zonation of phototrophic and autotrophic organisms (van Gemerden 1993, Stal 2000). Regarding

the air-dry habitat, the missing abiotic component and the 4- 5 times stronger degree of attenuation in the aeroterrestrial biofilm, we assume that it is highly compacted in contrast to aquatic mats.

Physiological adaptations in dependence of mixotrophy. Environmental conditions accountable for the change from autotrophy to heterotrophy are reduced radiation intensities and elevated substrate concentrations (Burkholder et al. 2008). Microalgal species capable of metabolizing exogenous organic compounds exhibit a range of responses that affect photosynthesis and growth. At one extreme are algae that totally repress chloroplast development ("bleaching") and change to heterotrophic nutrition exclusively, while on the other hand significant enhancement of pigmentation ("greening") accompanied by variation in photosynthesis and growth have been observed (Lewitus and Kana and references therein). Growth rate is believed to decrease under mixotrophic conditions, as the maintenance of two enzymatic systems is energy demanding. The here investigated *A. lobatus* strongly enhances its pigmentation, adapts the photosynthetic rate and maintains an equally low growth rate with, however, enhanced growth capacity due to high carbon concentrations in the mixotrophic medium. It is further able to survive long periods in absolute darkness without bleaching (data not shown), demonstrating the ability to grow purely heterotrophic.

We assume that the high glucose concentrations provided by the medium "imply" a microhabitat embedded in photosynthetically active (photoassimilate dispensing) cells and, thus, light limitation. Following that trigger, cellular Chl *a* concentration is strongly increased, supported by the additional exogenous energy supply (fig. 3) in order to maximize photon capture efficiency. However, Chl *a* in an excited state has the potential to form a triplet state, which can react with oxygen molecules in the vicinity to form singlet oxygen (Larkum 2003). Singlet oxygen is highly chemically reactive and damage can arise quickly. Within the so called "triplet valve", the triplet state of Chl *a* is passed on to an adjacent carotenoid which then decays by various mechanisms releasing heat (Frank and Cogdell 1996). The concomitant accumulation of lutein (Fig. 3B) can thus be interpreted as protection from reactive oxygen species. Photosynthetic rate is expressed as µmol O_2 h^{-1} mg Chl a^{-1} and displayed typical low-light adaptation with low compensation points at all investigated conditions. At 20 µmol photons $m^{-2}s^{-1}$ (Fig. 2A), photosynthetic rate can also be expressed on a "per cell" basis, as mixotrophic and autotrophic cells exhibit the same Chl *a* content under this conditions (Fig. 3). Thus, mixotrophic cells exhibit a two-fold lower photosynthetic rate which can be interpreted as metabolic shift towards heterotrophy. At high light conditions (Fig. 2B), mixotrophic algae exhibit a two-fold higher photosynthetic rate per Chl *a*. However, regarding the photosynthetic rate per cell, an equal rate at the two conditions is likely, as the Chl *a* concentration is generally increased in mixotrophic algae at comparable light intensity (fig. 3). The only remarkable difference between the two metabolic modes is the strong photoinhibition of autotrophic

cells at 30 µmol photons $m^{-2}s^{-1}$. This light-sensitivity is in accordance with the results of Gray et al. (2007) for desert green algae. He suggests that in the field algae are protected by complex spatial arrangements within the algal biofilm, resulting in adaptation towards low light conditions. We expand that theory by the impact of mixotrophy in natural biofilms which has rarely been considered in laboratory or field studies. It is likely that organic substrata are a ubiquitous component in all biofilms layers and we hereby demonstrate that organic substrate supply affects pigment concentrations and photoprotection *in vitro*. The xanthophyll cycle (e.g. Masojídek et al. 2004, Lawrenz 2005) enables algae to withstand excessive radiation. Autotrophic *A. lobatus* cultures showed the classical xanthophyll cycle with a 1:1 conversion of viola- to zeaxanthin (Fig. 4A). Glucose supply led to an increase in constitutive violaxanthin concentration and a doubling of zeaxanthin concentration due to high light conditions (fig. 4B). Thus, the xanthophyll cycle is evident in autotrophic algae but the enhancement of constitutive violaxanthin concentration (Fig. 4B) under mixotrophic conditions implies an improved photoprotection under exogenous glucose supply. Further, mixotrophic cells are effectively protected against reactive oxygen species by the "triplet valve" (see above).

However, all experiments have been conducted in liquid culture with high glucose concentrations and consequently, the results are not fully applicable to *in situ* conditions, where desiccation is an additional stressor and several organisms compete for the same substrate. Desiccated algae are more sensitive to photoinhibition as the pigments continue to absorb radiation while the chemical reactions of photosynthesis are inhibited and the excitation energy cannot be used for photochemical work. Gray et al. (2007) reported that desiccation combined with illumination led to recovery of only 50% of investigated desert green algal taxa, while without illumination all species recovered well. To finally evaluate the role of mixotrophy in physiological and ecological performance of *A. lobatus*, the factor desiccation needs to be included in the experimental set-up.

Mixotrophy in biofilms. Whether aeroterrestrial algae use dissolved organic substrates in nature is debatable. The demonstration of facultative heterotrophy in algae by routine methods is, at best, only regarded as a presumptive evidence for these mechanisms in nature (Parker 1961). Further, heterotrophic metabolism in algae is a rarely studied topic. Although ecological studies have provided insight into the capacity of algae to take up organic compounds, physiological studies on the interaction of heterotrophic metabolism and photosynthesis are so far restricted to marine organisms (Lewitus and Kana 1994, Tuchman 1996, Jones 2000). Nothing is known about the quantity and quality of organic matter in aeroterrestrial biofilms, although a high enzymatic activity has been shown for freshwater biofilms (Thompson and Sinsabaugh 2000) and is assumed for comparable aeroterrestrial biofilms (Karsten et al. 2007). Under natural conditions, algae have to compete with ubiquitous bacteria for

valuable organic substrates. Bacteria are regarded as the most efficient users of organic carbon and, hence, they typically act as competitors' superior to larger organisms (Wright and Hobbie 1966). Kamjunke et al. (2008) demonstrated that heterotrophic bacteria showed higher specific glucose uptake rates and outcompeted algae under dark conditions.

Microscale measurements of gross photosynthesis by oxygen microsensors (Revsbach et al. 1983, Revsbach and Jørgensen 1986) revealed a detailed spatial resolution of photosynthetic activity within aquatic microbial mats. Al-Najjar et al. (2009) presented the first complete energy budget assessment in a cyanobacterial mat, which, however differs in various features from the here investigated aeroterrestrial biofilm (see above). As no direct measurements of O_2 evolution have been performed during this study, theoretical assumptions correlating information about the aeroterrestrial habitat, results of light climate measurements and photosynthetic performance are presented. However, to clarify the importance of mixotrophy in deep biofilm layers, *in situ* measurements of O_2 gradients, quality and quantity of available substrates and enzymatic activities are required.

Competition and interaction in aeroterrestrial biofilms. The here investigated *A. lobatus* is a classical k-strategist with low growth and mortality rates, long lifespans and efficient resource utilization capacities (MacArthur and Wilson 1967). Raven (1997) calculated the "costs" of the photosynthetic apparatus to about 50% of the disposable energy while the phagotrophic apparatus accounts for only 10%. Consequently, *A. lobatus* can be classified as an organism, whose major mode of nutrition is autotrophy (as pigments are not reduced under prolonged dark conditions), while heterotropy may supplements metabolism under light-limitation. The use of several resources though with lower efficiency is discussed as an equal or even more successful competitive strategy than specialization (Tittel et al. 2003, Katechakis and Stibor 2006) and probably leads to the ecological success and natural dominance of *A. lobatus*. Nevertheless, to finally prove this hypothesis comparative studies on mixotrophy within other prominent aeroterrestrial taxa are essential. However, phototrophic biofilms are based on interactions between autotrophic primary producers and heterotrophic organisms. Diatoms in aquatic biofilms are known to enhance their EPS-production during co-cultivation with specific bacterial communities (Bruckner 2008). EPS excretion serves multiple important purposes in the life-cycle of aeroterrestrial green algae and the role of bacteria in that processes has not been discovered so far. Further, the question about lichen-like symbiosis in *Apatococcus* spp. needs to be resolved, as a close interaction with fungi (e.g. *Coniosporium*, observed by Turian 1979, Beck and Persoh 2009) and subsequent growth enhancement have already been described (Edlich 1936). Another evidence indicating mutualistic interactions between algae and fungi is the hydrophobicity of natural aeroterrestrial biofilms which is not evident for pure cultures of SAG 2096 (own observations) and arises probably due to the

existence of hydrophobins. These substances are produced by the mycobionts and surround the lichenized algae with a water repellent “coat”, facilitating the gas, water and nutrient exchange between the symbiotic partners (Dyer 2002). Further, the observed growth enhancement during co-cultivation needs to be quantified, possibly providing another evidence for positive species-interactions. Gorbushina et al. (2005) investigated the mutualistic interactions between microcolonial fungi and lichen photobionts *in vitro* which is another promising approach for *Apatococcus* spp., capable to finally resolve the question about lichen symbiosis.

Consequently, further research is required to investigate i) competition for resources with aeroterrestrial mixotrophs and heterotrophs ii) the identity of bacteria and fungi in aeroterrestrial algal-dominated biofilms and iii) the interactions between eu- and prokaryotic organisms. Due to the significant enhancement of growth capacity under mixotrophic conditions, laboratory work with *Apatococcus* is facilitated considerably and allows application of this successful aeroterrestrial alga within material research and testing designed to investigate biofouling problems.

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4. General Discussion

Algae are traditionally regarded as aquatic organisms and, compared to their total biodiversity, only a small fraction of them lives predominantly in terrestrial habitats (Ettl and Gärtner 1995, Lee 1999). The biodiversity of aeroterrestrial green algae in Germany has been analyzed intensively by Mudimu (2008). Consequently, the algae investigated in the present study were chosen due to their abundance in natural habitats. As the results presented here are part of an interdisciplinary project (designed for solving phylogenetical, distributional and ecophysiological questions concerning aeroterrestrial algal biofilms), the "linking" topics biodiversity and distribution are discussed first. The multiphasic study on *Chloroidum* (chapter 3.1, Darienko et al. 2010) contributes to that topic, as it clarifies the phylogenetic position of a many *"Chlorella"*-like aeroterrestrial green algae by the combination of molecular and physiological investigations. Following, chemotaxonomy is discussed as a link between molecular taxonomy and biochemical capabilities (chapter 3.2, Gustavs and Karsten, submitted). Further, the adaptation strategies of aeroterrestrial green algae are discussed in detail. The aeroterrestrial way of life requires a multitude of additional adaptation strategies including morphology, physiology (photosynthesis and growth) and metabolism (protective substances). The results focused on growth under water restriction (chapter 3.4, Gustavs et al. 2009b) and mixotrophic but light limited conditions (chapter 3.5, Gustavs et al., in prep.). Additionally, methodological aspects of growth rate measurements (chapter 3.3, Gustavs et al. 2009a) and their implications for natural distribution and measure of competitive strength have been evaluated. Finally, an outlook to future research options with special focus on integrated biofilm research is given.

4.1 Biodiversity and distribution of aeroterrestrial green algae

Species concept. Investigations of species diversity are inseparably related to a debate about the actual species concept in algae. This topic is intensively discussed and should build the basis for any considerations about biodiversity or distribution. For a long time, systematics and taxonomy of green microalgae were based entirely on morphological features. The *morphological species concept* defines species as groups of morphologically identical or similar organisms, while the *biological species concept* defines them as group of interbreeding populations which are reproductively isolated from other groups. The latter species concept is not valuable for many algal groups as they reproduce asexually by zoospore formation. The development of electron microscopy in the 1970ies resulted in many modifications and new insights concerning green algal systematics unravelling a wealth of ultrastructural features that were used as basis for a new classification (Mattox and Stewart 1984). In the 1990ies,

molecular systematics arose, which confirmed many conclusions based on electron microscopy, and which proved that in many cases the general morphology of green microalgae does not reflect phylogenetic patterns (Rindi et al. 2010 and references therein). Therefore, classification based on the morphological species concept is conversely debated, as molecular analyses demonstrated cases of close affiliation between morphologically highly divergent taxa and, contrary, examples of little relationship between morphologically similar taxa (Coesel and Krienitz 2008). The *phylogenetic species concept* is also hampered, as it is not clearly defined whether 'small' or 'large' genera better circumscribe the real natural lineages. The species concept is still "under construction" and unravelling the relations between individual lineages will need careful interpretation. The recent situation is characterized by the quest for a compromise between the conventional (morphological) and the phylogenetic system (John and Maggs 1997, Pröschold and Leliaert 2007, Coesel and Krienitz 2008, Luo et al. 2010).

Therefore, *multiphasic approaches* combining several research disciplines are regarded as good compromise in solving taxonomical uncertainties (Pröschold and Leliaert 2007, Coesel and Krientiz 2008, Darienko et al. 2010). The combination of molecular, morphological, physiological and chemical traits can lead to a valuable classification of certain strains to different genera or orders and, thus, to an improved species concept.

Biodiversity. The changes and compromises within the species concept complicate definite statements about biodiversity. Even though biodiversity estimations based on microscopical investigations imply a serious degree of uncertainty, several hundred aeroterrestrial species have been identified using unialgal cultures and classical microscopical techniques (Ettl and Gärtner 1995, Nienow 1996). Nevertheless, algae with characteristic morphotypes such as the Trentepohliales (Rindi and Guiry 2002, Rindi and López-Bautista 2008) and some Prasiolales (Rindi et al. 2004) could be identified reliably by light-microscopy of natural biofilms. However, microscopical identification of most unicellular aeroterrestrial green algae is difficult due to a high degree of morphological similarity (Rindi 2007 and references therein). Typically, they exhibit a small size (5- 50 µm) and only a limited number of representative characteristics. Their morphology is simple and usually referable to three habits: 1) single cells (*Chloroidium, Chlorococcum, Stichococcus, Trebouxia*), 2) sarcinoid habit, i.e. packet-like colonies (*Apatococcus, Desmococcus*) and 3) uniseriate filaments (*Klebsormidium, Rosenvingiella, Trentepohlia*) (Rindi et al. 2010). Furthermore, especial algae in natural samples exhibit thick cell walls and mucilaginous layers, complicating morphological indentification. It is still unknown if the simple and similar morphotype across many aeroterrestrial green algal taxa is related to adaptation to the terrestrial habitat (Karsten et al. 2007).

A prominent example for the phenomenon of "morphological convergence" is the *Chlorella*-morphotype, which appears in several distinct algal lineages, hiding a great diversity behind very similar morphologies (Lopez-Bautista et al. 2007). This morphotype exhibits small (< 15µm) spherical or elliptical cells, lacking characteristic morphological features. Taxonomical revisions (Huss et al. 1999, Krienitz et al. 2004) clarified the phylogenetic position of true *Chlorella* species (*C. vulgaris, C. sorokiniana, C. lobophora*) while all other *Chlorella*-like algae belong to different genera as *Parachlorella, Chloroidium* or *Scenedesmu*s. A high degree of "phenotypic plasticity" (variable morphology due to abiotic conditions) further complicates the taxonomy of aeroterrestrial green algae (e.g. Darienko et al. 2010). Recently, it has been realized that several morphological criteria represent phenotypical adaptations to ecosystem conditions and do not adequately reflect phylogenetic relationships (Krienitz et al. 2004, Luo et al. 2005, Luo et al. 2010).

Due to the presented taxonomical uncertainties, *culture-independent molecular approaches* are regarded as advantageous to unravel genetic diversity (Rindi 2010). The rRNA approach (Olsen et al. 1986) provides insight into the microbial world missed by traditional cultivation. Denaturing Gradient Gel Electrophoresis (DGGE) is based on differential electrophoretic mobility of PCR amplified DNA molecules in polyacrylamide gel with an increasing concentration (gradient) of denaturants, which detects single base changes in a segment of DNA (Fisher and Lerman 1983). This method is well established in biodiversity studies of cyanobacterial assemblages (e.g. Garcia-Pichel et al. 2001) and algal communities (Diez et al. 2001, Gast et al. 2004, Mudimu 2008 and references therein). DGGE provides a relatively fast overview ("fingerprint") about biodiversity in microbial communities and became one of the central tools applied in molecular microbial ecology. Gene cloning and sequencing (usually 18s rDNA) together with the construction of clone libraries determine the present biodiversity to a high resolution. Operational taxonomical units (OTUs) are grouped by distance-methods while phylotypes can be defined by parsimony-methods in sequence database analysis programs as ARB (Ludwig et al. 2004). The analysis of the internal transcribed spacer region (ITS rDNA) allows an even higher resolution of genetical differences (Mai and Coleman 1997), and hence, for example, further differentiates between cryptic species. However, there are well-documented limitations to the use of molecular methods in biodiversity studies such as the occurrence of PCR artifacts (von Wintzingerode et al. 1997, Qiu et al. 2001, Lawley et al. 2004). Furthermore, neither DGGE analysis nor clone libraries provide quantitative data and only give presence-absence records (Lawley et al. 2004). The "quality" of clone libraries is dependent on the quality of the sample data, the available reference data, and the discrimination accorded by the sequence used (Lawley et al. 2004). Reference data quality is often problematic as mislabeled and incorrect sequences appear in databases (Bridge et al. 2003), while the

proportion of organisms for which sequences are available is still relatively low. This is especial true for aeroterrestrial algae which are less abundant in such databases than aquatic algae or vascular plants. For example, by January 2009 very few sequences were deposited in GenBank for *Apatococcus* and *Desmoccus*, two of the most widespread genera (0 for *Apatococcus*, 5 for *Desmococcus*) (Rindi 2010). If available, the reference sequences should be determined from defined algal strains originating from professional culture collections.

Lewis and McCourt (2004) have dealt with the described classification problems by only presenting a “working classification of green algae and land plants” (Pröschold and Leliaert 2007), not intending to introduce a definitive taxonomic revision of green algal classification.

Besides morphological and molecular taxonomical markers, *chemotaxonomy* can be a useful tool to distinguish algal groups. The basic concept of chemotaxonomy is that various algal metabolites serve as an expression of genetic characteristics of distinct species. In algal taxonomy, the occurrence or lack of specific metabolites, such as pigments (van den Hoek 1993, Jeffrey and Vesk 1997), cell-wall carbohydrates (Takeda 1993), low molecular weight photosynthates (LMWC) (Kremer 1980, Karsten et al. 2007) or sterols (Görs et al. submitted), has been considered as highly useful for distinguishing groups at several taxonomical levels. Although the physiological state of an algal species and the environmental conditions strongly influence the intracellular concentrations of organic osmolytes (Karsten et al. 2005), the principal biochemical capability to produce particular compounds is useful for chemotaxonomical considerations. This is well documented within the morphologically simple, early diverging red algal lineages such as the Bangiophyceae, Porphyridiophyceae, Rhodellophyceae and Stylonematophyceae (according to Yoon et al. 2006), because each of these genetically well defined classes exhibits a distinct carbohydrate profile (Karsten et al. 1999, 2003). In that context, LMWC patterns in aeroterrestrial trebouxiophycean taxa have been analyzed comprehensively (Gustavs and Karsten, submitted). The results clearly demonstrate the chemotaxonomical value of specific polyols for aeroterrestrial Trebouxiophyceae.

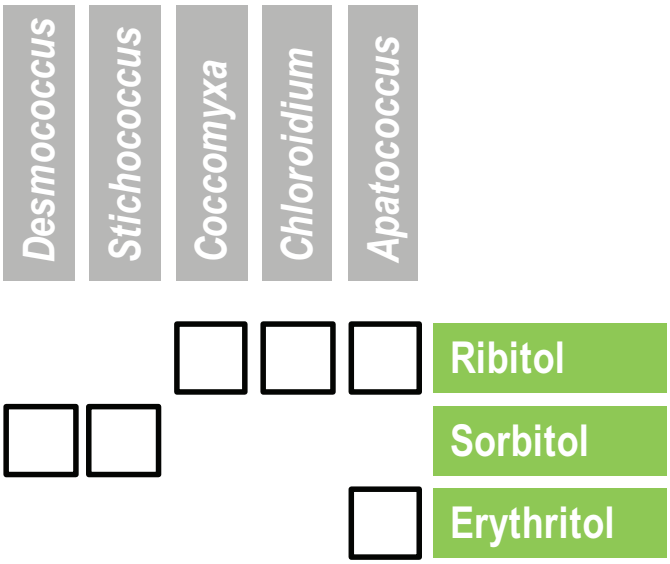

Fig. 4.1 Polyol pattern in abundant aeroterrestrial green algal genera (according to Gustavs and Karsten, submitted)

On the one hand, the polyol distribution helps identifying morphological simple coccoid green algae such as *Chlorella* and *Chloroidium.* As *Chlorella* is of great interest as model system for research and for biotechnological purposes, identification and classification of questionable strains can be supported by polyol analysis rather molecular investigations. The same is true for the sterol composition, which can also be used for chemotaxonomical purposes. Only *Chlorella*- and *Parachlorella*-species (Krienitz et al. 2004) contain ergosterol, while members of the *Chloroidium*- or *Scenedesmus*-clades lack this compound (Görs et al. submitted). Thus, two relatively simple and economic techniques are available to reliably distinguish between *Chlorella* and *Chloroidium*. Generally, the absence of a biochemical trait should not be used for taxonomic classification as it is possible that the concentration is too low or that accumulation up to measurable amounts proceeds under certain (unregarded) stress situations, i.e. that the substance is not constitutive. Therefore, an unidentified green alga is verified as *Chlorella* if it contains ergosterol, while the identity of *Chloroidium* is proofed by the presence of ribitol. The polyol analysis can also be assigned as quality assurance for purity of industrial scale *Chlorella* production in open ponds. As *Chloroidium* species are widely distributed in all types of habitats and are highly competitive, exhibiting fast growth rates under a wide range of abiotic conditions (Gustavs et al. 2009b, Darienko et al. 2010, unpublished results), it is possible that severe contamination of open *Chlorella*-production ponds can occur. If certain polyols are detected in measurable amounts in such pond samples, a contamination with other green algae or even fungi can be assumed (Lewis and Smith 1967). From an economic point of view, a chemical analysis is preferable against a molecular analysis, because the former is quick and easy to perform. Another aspect of this chemotaxonomical approach is the determination of biogeographical distributions of certain Trebouxiales by polyol analysis of natural biofilms (see following paragraph).

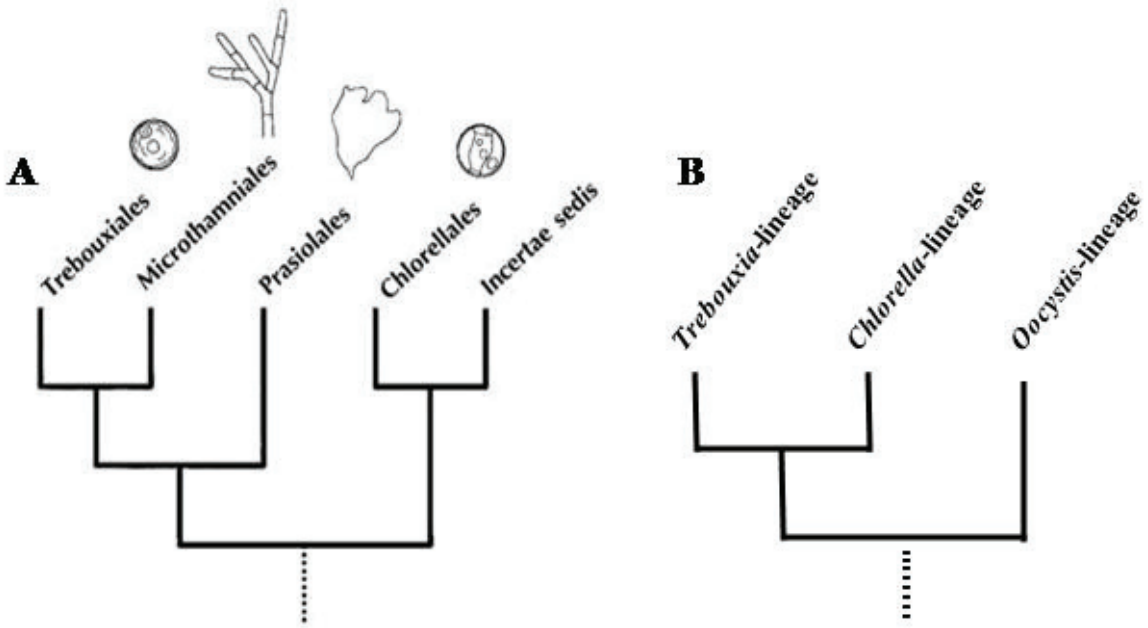

Fig. 4.2: Summary of phylogenetic relationships of major lineages within the Trebouxiophyceae based on 18S rDNA data. **A** according to Lewis and McCourt 2004, **B** according to T. Pröschold, pers. communication

Lewis and McCourt (2004) arranged the Trebouxiophyceae in five orders (Fig. 4.2 **A**), taking further major rearrangements into account as these algae are "referred to with some frustration as LRGTs, or 'little round green things' (Graham and Wilcox 2000)". The Trebouxiales are also named as the "lichen-algae group" due to typical photobionts such as *Trebouxia* or *Coccomyxa* and their predominant terrestrial distribution. However, the latter classification is irritating, as *Trentepohlia* (Ulvophyceae) and *Heterococcus* (Tribophytes) are also associated with lichens. The Prasiolales are commonly found in a great range of environmental conditions, including freshwater, marine, and terrestrial habitats while the Chlorellales seem to be comprised of freshwater species (Lewis and McCourt 2004). Recent molecular results based on rDNA data (T. Pröschold, personal communication) indicate a three-parted taxonomy within the class Trebouxiophyceae. The arrangement into the *Trebouxia*-, *Chlorella*- and *Oocystis*-lineage (Fig. 4.2 **B**) does not reflect official nomenclatural changes but will be used in the following to facilitate classification of the investigated Trebouxiophyceae.

According to that classification (and in contrast to Lewis and McCourt 2004), several distinct clades such as *Trebouxia, Prasiola, Watanabea* and *Microthamnion* are arranged within the *Trebouxia*-lineage (Fig. 4.3). The majority of related species and genera are either lichen symbionts (e.g. *Trebouxia*), exclusively aeroterrestrial (e.g. *Apatococcus*) or distributed in a great range of habitats (e.g. *Stichococcus* and *Chloroidium*). Apparently, all of them are able to withstand air-dry environmental conditions, although tolerance limits surely vary between species.

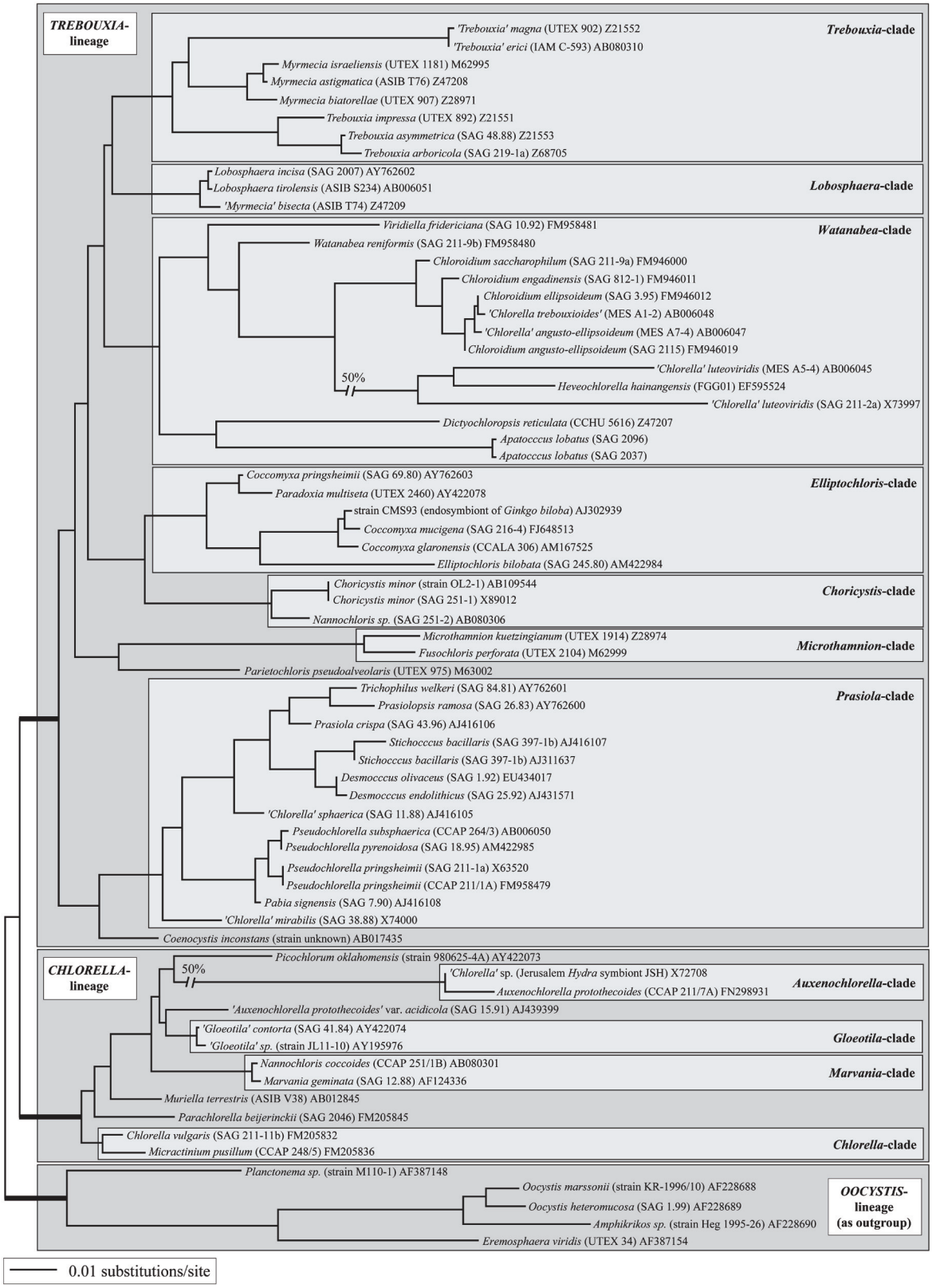

Fig. 4.3: Detailed phylogenetic tree of the *Trebouxia*-lineage based on 18S rDNA data (T. Pröschold 2010)

As mentioned before, the work of Mudimu (2008) has been part of this interdisciplinary project about "green algal biofilms on artificial hardsubstrates". The biodiversity results obtained within the cooperation influence the presented discussion and, thus, are summarized and discussed in the following section. The algal diversity in 79 green algal biofilms from different habitats (geographical provenance mainly Europe) has been investigated by 18S rDNA gene cloning and sequencing or DGGE fingerprint analysis. A total number of ~50 unialgal strains from the SAG culture collection have been sequenced as reference to the obtained results (O. Mudimu, pers. communication). The molecular data confirmed the dominance of the *Trebouxia*-lineage in aeroterrestrial habitats, while algae belonging to the *Chlorella*- or *Oocystis*-lineage were virtually not present. The filamentous green algae *Klebsormidium* spec. (Klebsormidiophyceae, Streptophyta) occurred frequently, representing the only representative from a different class. A total of 17 different OTUs in 7 major clades have been determined (Tab. 4.1). Furthermore, 9-17% of the clones belonged to the fungal classes basidiomycetes and ascomycetes (Hallmann 2007).

Tab. 4.1: Occurrence (%) of major algal species in 79 green biofilms (34 clone libraries and 43 DGGE fingerprints, according to Mudimu 2008), definition of OTUs and reference of actual species description

Class	Order	Clade	Occurrence (%)	OTU (operational taxonomical unit)	Reference
Trebouxiophyceae	*Trebouxiales*	*Apatococcus*	75	cryptic species: A, B, C, D, E	Hallmann et al. (in prep.)
		Chloroidium	61	*Cd. (angusto-)ellipsoideum* *Cd. saccharophilum* *Cd. engadinense*	Darienko et al. (2010)
		Jaagichlorella	27	A (SAG 2196) B (SAG 2213)	Darienko et al. (in prep)
		Prasiola	17	*Stichococcus* *Diplosphaera*	Dissertation L. Hodac
		Coccomyxa	11	*Avernensia* A *Avernensia* B *Pseudoccomyxa*	Friedl et al. (in prep.)
		Pabia	5	*Pabia* sp.	
Klebsormidio-phyceae		*Klebsormidium*	16	*Klebsormidium* sp.	Rindi et al. (2008)

In contrast to these molecular data, microscopical investigations revealed only 2-4 different morphotypes. Thus, the genetic diversity within the studied green algal biofilms exceeded microscopical observations by a factor of 5. Nevertheless, desert microbiotic crusts revealed a higher diversity, hosting

37 green algal taxa from 19 genera belonging to the classes Chlorophyceae, Trebouxiophyceae and Charophyceae (Flechtner et al. 1998). The biodiversity of investigated biofilms is definitely higher than determined by microscopical observations, but less pronounced than in other terrestrial habitats. The most abundant species is *Apatococcus* which has been confirmed by molecular techniques in 75% of the investigated biofilms (Tab. 4.1). Additionally, microscopical investigations frequently revealed quantitative dominance of *Apatococcus* in aeroterrestrial biofilms (Gustavs et al. in prep., Görs et al. in prep.). Gärtner and Ingolic (1993) assumed that the genus *Apatococcus* consists of a single variable species only, *A. lobatus*. To unravel the diversity within the *Apatococcus*-clade, the internal transcribed spacer region (ITS 1,2 rDNA) has been sequenced, revealing the existence of five well-supported OTUs (Hallmann et al. 2010 in prep.), which, however, was not supported by 18S data or morphology. Comparison with biofilms from tree barks and needles from *Picea abies* (U. Søchting, Copenhagen) revealed the same cryptic diversity within a single habitat. A possible explanation for the phylogenetic diversity is a functional separation into ecotypes: each of the OTUs would than dominate the community under certain (seasonal characteristic) conditions. Even though ecotypes can display morphological, ecological or genetical differences, they have no formal taxonomical position. Their formation could proceed through geographical isolation (Boenigk et al. 2007) but also through selective pressure within a spatial well defined habitat. Further investigations concerning ecophysiological characters as growth or metabolic performance under varying abiotic conditions are necessary to clarify the question, if the detected *Apatococcus* OTUs belong to ecotypes, “cryptic” or “real” species.

The second-most frequent genus, *Chloroidium,* has been detected in 61% of the investigated sites. These *Chlorella*-like ellipsoidal green algae belong to the *Watanabea*-clade, while *Chlorella vulgaris* is part of the *Chlorella*-clade (Darienko et al. 2010). *Chloroidium, Stichococcus* and *Jaagichlorella* occur in all kinds of habitats and seem to exhibit a cosmopolitan distribution (Lewis and McCourt 2004, Darienko et al. 2010). Within a monthly sampling campaign (02/2007-05/2008), the question about seasonal succession in green algal biofilms was evaluated by a combination of molecular and microscopical investigations. Preliminary results demonstrate that no successive shift in community structure proceeds and *Apatococcus* dominates the community throughout the year (Görs et al. in prep.).

An important aspect of microbial ecology is the phenomenon of “unculturable” microorganisms. The so called “Great Plate Count Anomaly” (Staley and Konopka 1985) describes the discrepancy between microscopical cell counts in the natural inoculum (high) and colony count in the Petri dish (low). Initially, the discrepancy has been explained by limits in mimicking natural conditions in the laboratory. Although this anomaly has been defined for bacterial communities, it is also evident in the here investigated algal assemblages. While *Apatococcus* is abundant in natural assemblages, it disappears frequently during raw cultivation. In contrast, *Chloroidium* is rarely visible in natural samples, but omnipresent in culture-

independent approaches and after raw-culturing of biofilm material. Thus, raw-culturing discriminates aerophilic algae perfectly adapted to the existing natural conditions, while it privileges species merely bearing those harsh conditions. Consequently, a shift in community structure proceeds due to the artificial culture conditions. Even when plated on solid media (agar) which better mimics natural growth conditions, *Apatococcus* is not able to compete and is rapidly overgrown by other species. The different lifestyles and survival strategies are illustrated by growth rates under standard culture conditions (Fig. 4.4 A). *Apatococcus* is classified as a K-strategist with low growth and mortality rates, long lifespans and efficient resource utilization capacities while *Chloroidium* is a r-strategist characterized by high growth and reproduction rates (MacArthur and Wilson 1967, chapter 3.5).

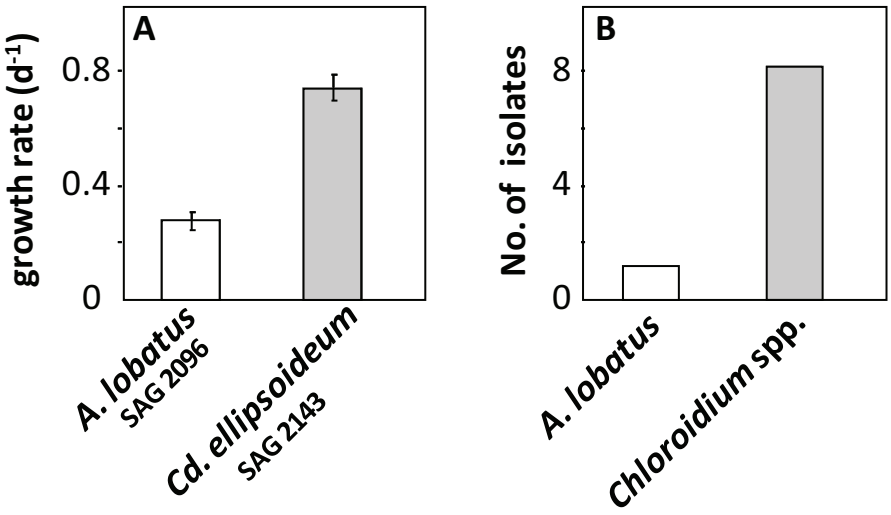

Fig. 4.4 **A** growth rate (d^{-1}) at 20°C and 50 µmol photons $m^{-2}s^{-1}$, n=3 and **B** number of isolates obtained during first isolation-campaign within the project

Other aeroterrestrial genera, such as *Stichococcus*, *Coccomyxa* or *Jaagichlorella* exhibited two- or three-fold higher growth rates under the described culture conditions (data not shown, but see Gustavs et al. 2009b). The difficulties in cultivation are reflected by the low number of isolates obtained (Fig. 4.4 B). Extensive variation in culturing conditions (solid, liquid or substrate-enriched media, temperature between 0-30°C, PFDs between 10 and 300 µmol photons $m^{-2}s^{-1}$) did not result in higher growth rates of *Apatococcus*. Consequently, the ecological success of this abundant aeroterrestrial green alga does not originate from competitive strength based on growth (and resource-utilization) rate but obviously from long-time survival under harsh environmental conditions. Generally, *Apatococcus* is regarded as "complicated to culture" (e.g. Edlich 1936, Gärtner and Ingolic 1989, Freystein et al. 2008) and probably some important (but still unregarded) factors explain its natural abundance (for additional discussion see chapters 3.5 and 4.3).

The prior paragraphs have illustrated the necessity for interdisciplinary research to improve the understanding of natural microbiotic communities. An exclusively culture-independent study gives information about qualitative biodiversity, while microscopical investigations provide knowledge about quantitative community composition which, however, could not be correctly determined due to the mentioned morphological difficulties. Raw-cultivation, isolation of species and determination of growth rates under controlled conditions give a (theoretical) insight into competitive strengths and limitations of specific genera. Nevertheless, the abstraction from laboratory results back to natural systems is extremely difficult and should ideally be combined with *in situ* measurements of metabolic activities and species interactions (competitive, mutualistic or symbiotic).

Distribution. Evaluating the biogeography of aeroterrestrial green algae remains problematic due to various reasons. The distribution of biofilms is characterized by a great variability at several spatial scales and is regarded as very irregular, depending on a complex interaction of numerous factors (Rindi 2007 and references therein, Chapter 1). Typically, phototrophic biofilms are dominated by unicellular algae, associated with amoebae, bacteria, fungi and inorganic particles (Gaylarde and Morton 1999, Gorbushina 2007). In temperate regions, such as North-Western Europe, green biofilms are dominated by eukaryotic green microalgae (mainly Chlorophyta, sometimes Streptophyta) (Rindi and Guiry 2003, Barberousse et al. 2006, Karsten et al. 2007, Mudimu 2008), whereas in warm-temperate to tropical regions they are mainly composed of cyanobacteria such as *Gloecapsa, Phormidium* or *Scytonema* (Ortega-Calvo et al. 1993, Gaylarde and Gaylarde 2000, Tomaselli et al. 2000). Diatoms (Bacillariophyceae, Ochrophyta) also occur in terrestrial habitats but are usually restricted to environments very close to the ground surface (own observations), habitats in caves (hypogean) or inside natural rocks (chasmolithic) (Gorbushina 2007 and references therein).

The geographic distribution and diversity of microorganisms, which are ultimately linked to each other, were controversially debated in recent years (Finlay 2002, Foissner 2008) despite, or perhaps because of, a limited understanding of the determining processes (Weisse 2008). In general, eukaryotic microbial species or protists (protozoa and protophytes) are supposed to be ubiquitous and to have a cosmopolitan distribution. Beijerinck's ubiquity hypothesis (see Brock 1966), taken up by Baas-Becking (1934) and finally accentuated by Finlay (2002) states that 'in micro-organisms, everything is everywhere, but the environment selects'. This statement resulted in an intense discussion focusing on the species concepts (see chapter 1). Recently, numerous articles have been published which contradict the ubiquity hypothesis (e.g. Foissner 2008). Accordingly, the discussed hypothesis is actively debated for microalgae (O´Malley 2007, Coesel and Krienitz 2008).

The previous detailed discussion about the species concept illustrates the main objection of the *ubiquity hypothesis*: morphological convergence and phenotypic plasticity complicate morphological species determination to such a serious degree, that misidentifications and erroneous "ubiquity" reports are the only logical consequence. Coesel and Krienitz (2008) evaluated biogeography of desmids, which build the "geographically best studied group of green algae due to their often appealing appearance", facilitating species determination. They concluded that cosmopolitan species are relatively scarce, although microorganisms in general can be readily distributed owing to their minute size. Another important aspect in that discussion is the recent extension of species related to climate change (e.g. *Micrasterias americana,* Coesel and Krienitz (2008)). The quantitative occurrence of aeroterrestrial green algae is also favored by mild and humid weather conditions (Gladis et al. in prep.). Concrete consequences of climate change surely differ from species to species: endemic psychrophyilic species may be extinct while euryoecious species will be able to disperse to formerly hostile habitats. However, if a qualitative change in species composition occurs in temperate green biofilms as consequence of climate change needs to be investigated.

The *moderate endemicity model* by Foissner (2008) is in conflict with the *ubiquity hypothesis*. The main contradicting point is the short generation time of microorganisms, promoting mutations and thus speciation. Accordingly, local and regional endemics should be widespread (Mann and Droop 1996). Further, Foissner (2008) stated that undersampling and misidentifications complicate the proof of both theories. These specific problems have been outlined recently for aeroterrestrial algae (López-Bautista et al. 2007, Rindi 2010).

In conclusion, it is not possible (or at least very difficult) to proof the presented hypotheses. Some aeroterrestrial genera seem to be cosmopolitan or at least wide spread due to their euryoecious characteristics (e.g. *Apatococcus* and *Chloroidium*) while other genera seem to have their centre of abundance and diversity in relatively restricted areas (e.g. Trentepohliales in the tropics, Rindi and Lopez-Bautista 2008). This is in agreement with numerous literature cites (Rindi 2007 and references therein). However, the question if elder (microscopy-based) literature correctly identified morphologically identical taxa such as *Desmococcus* and *Apatococcus* is hardly to be solved. Both taxa have been summarized within the "proto-pleurococcoid assemblage", although they are genetically well apart and probably display different growth optima and ranges. However, both genera seem to be restricted to terrestrial habitats which facilitate biogeographical investigations. In contrast, *Chloroidium* is widespread throughout all climatic zones and additionally able to colonize terrestrial, marine and freshwater habitats (Darienko et al. 2010).

4.2 Adaptation strategies of aeroterrestrial green algae

General remarks. Overall, aeroterrestrial microorganisms can be regarded as euryoecious (Gorbushina 2007, Karsten et al. 2007), that means they tolerate multiple and variable abiotic stresses and can cope with a combination of diverse environmental challenges. Although other habitats are characterized by more extreme conditions of temperature, pH and salinity, they are rarely subjected to such rapid and extreme fluctuations in environmental conditions as terrestrial ones (Gorbushina 2007). Terrestrial microbial mats inhabited by eukaryotic algae and cyanobacteria occur even in semi-arid regions and deserts throughout all climatic zones (Friedmann 1982, Palmer and Friedmann 1990, Bell 1993, Hoppert et al. 2004). These communities survive extreme temperatures, long desiccation periods and radiation conditions at both extremes (limitation in endo- or hypolithic habitats, excess in biological crusts). Investigations of such abiotic extremes are often motivated by theoretical assumptions about evolution of (extraterrestrial) life, as the processes of successful microbial exploitation of a novel habitat in otherwise harsh environments might be comparable (e.g. Schlesinger et al. 2003).

In contrast to terrestrial environments, aquatic habitats offer more stable physical conditions buffered through the aqueous environment. Water, the key factor for life, is never limited although variable salinities imply an additional stressor and the need for effective osmotic adaptation. While the growth limiting factor in many terrestrial ecosystems is water availability, in aquatic ecosystems availability of light is the most important factor regulating carbon fixation (Dubinsky and Schofield 2010). Light intensity decreases exponentially with depth and is additionally “spectrally skewed”, that means specific wavelengths are attenuated to different degrees. Therefore, most of the water column has insufficient light to support photosynthesis (Morel 1974, Mobley 1994). Even though mild and stable conditions are the norm for aquatic ecosystems, some habitats as tidal flats and mangroves face highly fluctuating abiotic conditions similar to the terrestrial biofilms discussed here.

In this study, aeroterrestrial green algae from temperate climatic zones were investigated. Naturally, the range of stressors is not comparable to aeroterrestrial habitats in hot or cold deserts as mentioned above. Due to humid summers and mild winters in mid Europe, the distribution and quantity of green biofilms on anthropogenic substrates has increased (Karsten et al. 2005, Gladis et al. in prep). Consequently, they attracted scientific interest from an applied point of view as they cause discolorations and even biofouling problems on such surfaces (e.g. Karsten et al. 2005, Eggert et al. 2006, Rindi 2007). However, the ecophysiology of aeroterrestrial green algae is an interesting research area in its own right, with numerous fundamental questions yet to be solved. The adaptation strategies of aeroterrestrial green algae to isolated and combined stressors are outlined and discussed in detail in the following part, with a focus on the recently obtained results.

Morphological adaptations. Aeroterrestrial green algae exhibit a very simple and uniform morphology. A small coccoid morphotype is regarded as fundamental advantage to survive water deficiency, because a small surface to volume ratio retards evaporation. Further morphological adaptations aiming the same purpose include thick cell walls (Fig. 4.5 A), colony formation (Fig 4.5 B) and mucilaginous layers.

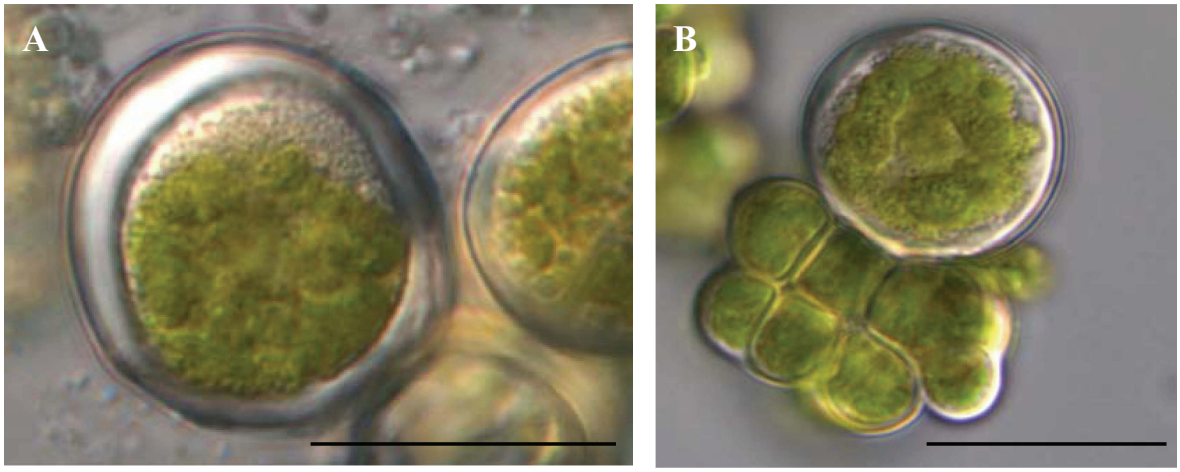

Fig. 4.5: **A** natural sample of *Apatococcus* exhibiting thick cell walls and **B** *A. lobatus* SAG 2096, cultured material building typical sarcinoid colonies *Scale bar*: 20µm (© C. Hallmann, Göttingen)

Another strategy to prevent desiccation is growth on or within particulate substrata (Friedmann 1980, de Winder et al. 1990) or the inclusion in a complex, pseudo-multicellular structure such as an algal mat (e.g. Stal and Caumette 1993) or the thallus of a lichen (e.g. Nash 2006). Any of these structures serve as water reservoir and help to retard evaporation and, possibly, to condense water at high humidities (Nienow 1996). In addition, growth in larger assemblages such as biofilms generally provides physical protection too, for example, by the matrix of extracellular polymeric substances (EPS).

However, prolonged desiccation stress results finally in dehydration of algal cells. Under such conditions, spores (i.e. resting stages) are formed to cope with the environmentally unfavourable period (Karsten et al. 2007). Spores are characterized by thick, impregnated cell walls, which guarantee longterm survival in dry habitats. After contact to fluid water, they germinate within a short time initiating growth of a new algal population. The formation of unequal autospores is a characteristic feature for the genus *Chloroidium*, which is also regarded as a morphological adaptation giving competitive advance in terrestrial environments (Darienko et al. 2010). Small autospores can be easily distributed by wind or rain, while larger autospores have a better chance to become reproductive in the actual habitat. Therefore, dispersal is facilitated and continuity in already colonized habitats is assured.

Biofilms are stabilized by a matrix of EPS excreted by the colonizing organisms (Karsten et al. 2007). These biopolymers are hygroscopic and their presence creates a more hydrated

microenvironment in the immediate vicinity of the algal cells, thereby limiting desiccation stress (Potts 1994, Ophir and Gutnick 1994, Chang et al. 2007). The mucoid matrix of biofilms builds a diffusion barrier for nutrients and gases. While carbon dioxide should be easily provided by heterotrophic organisms associated with the algae (Kühl et al. 1996), it is unknown so far, if bacteria and fungi remineralize organic substrates sufficiently. However, a high enzymatic activity has been shown for freshwater biofilms (Thompson and Sinsabaugh 2000) and efficient remineralization within terrestrial biofilms is assumed (Karsten et al. 2007).

The synthesis of mucilage has various purposes: it serves as a water reservoir (Nienow 1996) and as a vector for exchange of compatible solutes or excessive photosynthates. Further, EPS facilitate interaction and communication between organisms and makes e.g. rock surfaces more receptive to further biofilm development and subsequent colonization (Gorbushina 2007). The establishment of aeroterrestrial biofilms is achieved by algal attachment via a two-stage process, initial and permanent adhesion, mediated by the secretion of EPS (Fletcher and Callow 1992, Mostaert et al. 2009). These processes are comparable with those in well-studied marine algae. Mostaert and co-authors (2009) characterized the nanostructure of algal adhesives, namely of *Chloroidium ellipsoideum* (SAG 2143) and *Coccomyxa* spec. (SAG 2040). The adhesives of the investigated green algae exhibited nanoscale repetitive sawtooth response to a tensile force as those measured for adhesives from multicellular subaerial algae, diatoms, a parasitic flatworm, and a bacterial a biofilm (Mostaert et al. 2009 and references therein). These results suggest a generic link between aqueous and terrestrial adhesives occurring in numerous organisms of phylogenetically different provenance. The abiotic conditions affecting algal attachment to terrestrial surfaces have not been investigated so far. However, it is assumed that pioneer colonizers on exposed surfaces are chemo-organotrophs that entrap minerals and organic substances from the air (Viles and Gorbushina 2003). Heterotrophic organisms are now accepted as important, sometimes dominant and occasionally pioneer settlers on mineral surfaces (Gorbushina 2007 and references therein). Identification of substrate-related factors (pH, roughness, hydrophobicity) affecting attachment is a useful link to applied research aiming prevention of biofilms on anthropogenic hard substrata.

Physiological adaptations. *Water* is an essential prerequisite for life on earth and the fundamental component in enzymatic processes and, thus, metabolic activity. Water restriction is therefore the key factor controlling algal growth in terrestrial habitats. Higher terrestrial plants form complex multicellular structures with an essentially water-tight surface to extend their period of activity. This option is not open to unicellular life forms, although an almost constant water concentration (homeostasis) is as well crucial

for cell vitality. Cellular dehydration (matric stress) can inhibit photosynthesis (De Winder et al. 1990, Gray et al. 2007), oxidise proteins, DNA and membrane components (for review see: Potts 1999). Dried green algal biofilms are physiological inactive but photosynthetic activity recovers rapidly after rewetting (Häubner et al. 2006), demonstrating the survival of aeroterrestrial biofilms under temporal water deficiency. The same is true for biological soil crusts, which are able to quickly restore respiratory and photosynthetic capacity and only metabolize actively after moistening (Lange 2001). This author further stated, that desiccated poikilohydrous organisms such as lichens are dormant and, in this state, highly resistant to challenging environmental conditions as extreme temperatures or excessive radiation. The ability to directly absorb air humidity is a key competence of aeroterrestrial algae and lichens, and is discussed as a fundamental prerequisite to survive in air-dry environments (Nienow 1996, Lange 2001). *Apatococcus* (Bertsch 1966), *Chloroidium, Stichococcus* and *Coccomyxa* (Gustavs et al. 2009b), *Trebouxia* (Palmer and Friedmann 1990), *Klebsormidium* (Karsten, unpublished data) and *Trentepohlia* (Ong et al. 1992) are able to grow without liquid water by uptake of water vapour. *Apatococcus* and *Trebouxia* were even able to photosynthesize well below of an air humidity of 70%. In contrast, cyanobionts or the mat-forming cyanobacterium *Microcoleus sociatus* need liquid water for positive net photosynthesis (Lange et al. 1986, Lange et al. 1992). The ability to use water vapour for photosynthetic productivity favours the existence of eukaryotic microalgae over cyanobacteria in coastal areas with low precipitation but frequently elevated air humidity. Air humidity in coastal areas transports sea salt, and microorganisms colonizing these habitats face *osmotic stress*. Lichens (with trebouxiophyceaen photobionts) growing close to the littoral zone are able to tolerate hyperosmotic stress up to marine levels. In contrast, inland species are less tolerant demonstrating that local adaptations took place rather rapidly (Nash and Lange 1988). The humid climate characteristic for Ireland is also affected by marine salt entry. The local algal biofilms are dominated by Prasiolales (Rindi et al. 2007) whose osmotic stress resistance has been shown by Jacob et al. (1991). Other prominent taxa in Irish aeroterrestrial biofilms are Trentepohliaceae which are closely related to marine seaweeds (class Ulvophyceae). Naturally, they exhibit a broad tolerance to salinity stress. Osmotic stress tolerance was also shown for free-living Trebouxiophyceaen such as *Coccomyxa* sp., *"Chlorella" luteoviridis*, *Apatococcus lobatus* and *Chloroidium ellipsoideum* (Gustavs et al. 2009b) which grew in a broad salinity range between 0 and 40-65 psu, achieving high relative growth rates (80% of the maximum) even under marine salinity conditions (28-47 psu). The implications of polyol synthesis and accumulation for osmotic stress resistance will be discussed in the following paragraph about biochemical adaptation strategies.

Radiation is another important variable for phototrophic organisms, controlling photosynthesis and, thus, the process of carbon fixation and its conversion into living biomass. Photosynthesis responds

quantitatively to changes in irradiance. Acclimation processes are central for aeroterrestrial microalgae as the range of insolation spans 4 orders of magnitude, from the maximal solar irradiance with 2374 µmol photons $m^{-2}s^{-1}$ (at local noon, Mobley (1994)) to low light conditions within a natural biofilm. A central benefit of colony formation is the self-protection against excessive radiation, as outer layers absorb harmful doses, shielding inner cells effectively from photodamage (Franklin and Larkum 1997, Stal 1995). Irradiance is attenuated exponentially after passing the outermost cell layers of aeroterrestrial biofilms: after 0.1 mm 90% of the ambient light is absorbed by cell layers, while at 0.2 mm only 1% is left (chapter 3.5). The situation in aeroterrestrial biofilms is distinct from biotic crusts, sediments or aquatic microbial mats, as virtually no abiotic particles scatter the incident light. Scattering and attenuation of ambient light in biofilms depends on compactness, which is in turn influenced by water content, biotic and particle composition. Therefore, the photic zone (defined by 1% intensity of ambient light) is much thicker in biofilms influenced by sediment particles and can reach up to 3 mm (Garcia-Pichel and Bebout 1996). Many phytoplankton and microphytobenthic species are capable of vertical migration, thus actively entering optimal radiation areas. Further, spatial zonation i.e. occurrence of different algal species in certain biofilm layers according to their growth optima, is commonly observed in marine microbial mats (Oren et al. 1995). However, active movement is not known for aeroterrestrial microalgae, and cells once “buried” at specific positions may suffer from radiation deficiency. An important biochemical adaptation to light limitation is mixotrophy, which enables primary photosynthetic organisms to supplement energy and carbon demand by organic substrate uptake (Sanders et al. 1990, Cloern and Dufford 2005). Thus, spatial zonation in aeroterrestrial biofilms can also be realized by functional layers (from auto- to mixo- and heterotrophic levels with increasing depth) as suggested in chapter 3.5 and fig. 4.7. However, mixotrophy *in situ* has not been investigated so far.

Besides light deficiency in inner biofilm layers, excessive radiation is a serious stressor to outer biofilm layers, which may damage the PSII D1 key protein (Aro et al. 1993). This in turn typically leads to decreasing photosynthetic rates (Henley et al. 1991). The inhibition of photosynthesis is often associated with the formation of reactive oxygen species (ROS) that interact with lipids, membranes and proteins to cause irreversible damage to a cell (Falkowski and Raven 2007, Dubinsky and Schofield 2010). Short wavelength radiation (UVR; 100-400 nm) with high energy content affects biomolecules such as nucleic acids and proteins (Vass 1997). Consequent photodamage or conformational changes to important cellular components in algal metabolism may result in reduced photosynthetic and general metabolic activity leading to a decrease or inhibition in growth and cell division (Franklin and Forster 1997). Green algae of the class Trebouxiophyceae actively regulate their absorption cross section and their internal light-harvesting pigments in response to excessive radiation (Lawrenz 2005). They further

possess the xanthophyll cycle and are able to quench excessive radiation by non-photochemical processes. The xanthophyll cycle is regarded as effective photoprotective mechanism against excessive PAR (e.g. Masojídek et al. 2004), while synthesis and accumulation of UV-absorbing mycosporine-like amino acids (MAAs) constitutes protection against UVR (Reisser and Houben 2001, Karsten et al. 2007).

Temperature affects, though in varying degrees, all metabolic and physiological processes. All enzymatic catalyzed reactions are temperature-dependent and, consequently, the rate of biological, biochemical, and physiological processes too. Reaction rate is generally doubled with a temperature increase of 10 K (RGT rule, Van't Hoff 1884). In this case the Q10-value of a reaction, i. e. the factor of acceleration, is two. Physiological temperatures range between 1 and 45°C, as below this range cellular water freezes and fluidity of lipid membranes decreases. Formation of intracellular ice crystals results in mechanical damage of membranes and cellular compartments, and hence an increase in mortality. Temperatures above 45°C lead to denaturation of enzymes. Growth in dependence of temperature follows a bell-shaped curve, characterized by a progressive increase in growth rate up to an optimal temperature, beyond which the growth rate rapidly declines (Blanchard et al. 1996, Fig. 4.6).

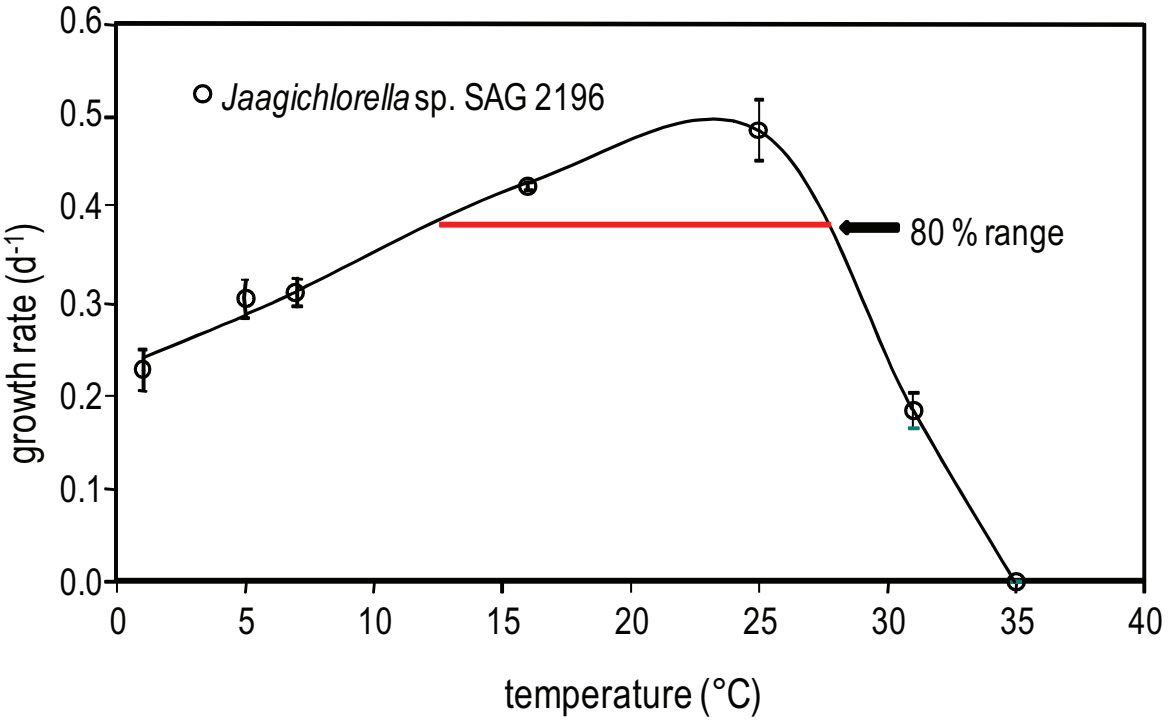

Fig. 4.6 Representative temperature-growth response of aeroterrestrial green algae. The absolute temperature range exhibits 30K, while the range of "preferendum" (80% of μ_{max}) is 14K wide (Häubner et al. 2006, optimum curve according to the nonlinear model of Blanchard et al. (1996)).

Further, the temperature range of *good* (80% of maximal rate) and *bad* (20%) growth can be calculated from the data fit (Darienko et al. 2010). The aeroterrestrial algae investigated so far are all characterized as eurytherm, growing well between 12 and 22°C with an optimum around 17-20°C (Häubner et al. 2006, Darienko et al. 2010, unpublished data). The ability to grow at higher temperatures is regarded as an evolutionary conservative character. This trait can even be used to support species determination, as demonstrated by Darienko et al. (2010). However, good growth is restricted to moderate temperatures. To discuss competitive strength under natural conditions, interactions between abiotic factors must also be considered. The activity of RuBisCo is temperature dependent, while the photophysical reactions providing electrons (e.g. light absorption) are not (Falkowski and Raven 2007). This results in an increase of radiation stress under low temperatures. Maxwell et al (1994) determined a five-fold decreased Chl *a* concentration in 5°C grown *Chlorella vulgaris*, combined with an increased xanthophyll concentration and a two-fold elevated Chl *a/b* ratio (compared to the control grown at 25°C). These temperature effects in algal metabolism are comparable to those reactions upon increased radiation (Davison 1991, Maxwell et al. 1994).

In this study, the *growth rate* has been chosen to assess algal responses to varied environmental conditions (Gustavs et al. 2009 a, b, Darienko et al. 2010). Growth rate, in contrast to other cellular processes such as photosynthesis, represents the most relevant process to describe the potential performance of species in their respective habitat as it typically characterizes ecological success. Consequently, growth rates also reflect life strategies of microorganisms (see Fig. 4.3) and are regarded as a feasible approach to evaluate competitive strength. However, it must be taken in consideration that any laboratory experiment permits only limited conclusions as difficulties in mimicking natural conditions are well known (see paragraph about “unculturable” microorganisms). Typical culture media provide high concentrations of anorganic nutrients, while organic substrates are usually not supplied to suppress bacterial growth. In phycological research, it is common to use unialgal and axenic cultures to exclude bacterial activity in e.g. photosynthesis measurements based on O_2 evolution. However, there are numerous examples of close interactions between autotrophic and heterotrophic partners (Mouget et al. 1995, Watanabe et al. 2005, Grossart et al. 2005, Bruckner et al. 2008) which are not considered by commonly applied approaches. The observed effects were often ambiguous. Growth of diatoms can be enhanced or suppressed by bacteria (Grossart and Simon 2007, Grossart et al. 2006); also the strength of adhesion to a substratum can be increased (Grossart 1999) or reduced (Wigglesworth-Cooksey and Cooksey 2005). It is generally accepted that the interactions between algae and bacteria are species-specific and, thus, can be negative or positive for growth and reproduction. So far, green-algae and

bacteria have rarely been cultivated in defined co-cultures and until now neither bacterial composition nor species-interactions have been investigated in aeroterrestrial biofilms.

Due to the described isolation and culturing customs, the capacity for *mixotrophy* has been "discovered" during purification procedure of xenic algal strains. Axenity is controlled by cultivation on substrate-rich media, where remaining bacterial contamination is rapidly visible. However, as massive algal growth and pigment accumulation was evident at respective test medium, the investigations about mixotrophy in *Apatococcus* were initiated (chapter 3.5). The strongly enhanced accumulation of biomass after organic carbon supply is a positive secondary effect, as *Apatococcus* was traditionally classified as "difficult-to-culture" and consequently unsuitable for routine investigations or as model organism (e.g. Freystein et al. 2008).

As suggested in the discussion of chapter 3.5, *in situ* measurements of photosynthetic and enzymatic activity are required to finally evaluate the ecological role of mixotrophy. Nevertheless, to illustrate the theoretical considerations about spatial differentiation within aeroterrestrial biofilms, a schematic drawing of biofilm zonation is presented in figure 4.7.

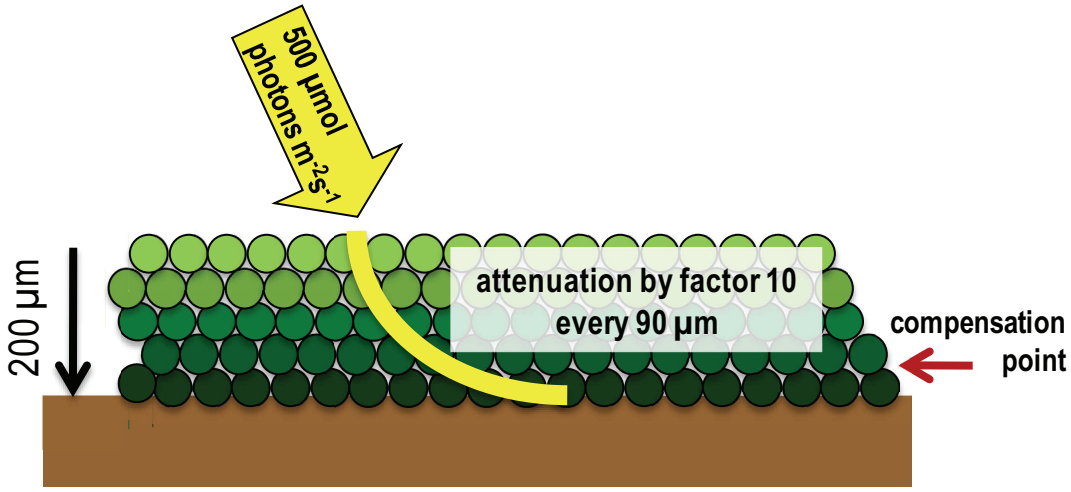

Fig. 4.7: Schematic drawing of biofilm architecture, distribution of metabolical processes and light climate in different layers.

Exposed biofilms on e.g. roof tiles are very thin (probably only a few µm, own observations) and, thus, experience high light conditions rather light limitation as suggested for the present case. Arboreal biofilms are usually not exposed to full sunlight, as they grow at a vertical substrate under the canopy of surrounding vegetation and are preferentially orientated north-wards (Nienow 1996). Though, the maximal PFD experienced by algae in such biofilms accounts for 500 µmol photons $m^{-2}s^{-1}$. If PAR is further attenuated with a factor of 10 every 90 µm, only the upper ~160 µm of the biofilm are supplied with sufficient radiation for net photosynthesis. Below that mark, the PFD equals the compensation point

(5-10 µmol photons $m^{-2}s^{-1}$) and photosynthesis would be negligible. In the discussed scenario, intermediate biofilm layers between 50 and 150 µm depth receive ideal radiation intensities. We further assume that algae beneath the 160 µm level meet their energy demand exclusively through mixotrophy, as light is definitely unsufficient for photosynthesis. Further, organic carbon supply should be increased with biofilm depth due to degradation processes, supporting mixotrophic growth. *Apatococcus* survives *in vitro* up to 4 month of complete darkness (own observations) and even pure heterotrophic layers at the base of biofilms are likely (Tuchman 1996).

The natural dominance but pronounced slow growth rate of *Apatococcus* contradicts the above discussed theory, that growth rate is a good measure for competitive success. As mentioned in chapter 3.5, *Apatococcus* is a slow-growing k-strategist who investigates its metabolic energy into long-time survival rather than into high reproduction and growth rates. Mixotrophy and survival under light-limited conditions is a possible explanation for its abundance which, however, may also originate from the absence of grazing pressure. *Apatococcus* cells are significantly larger than *Chloroidium* or *Stichococcus* cells, possibly preventing consumption by epiphytic grazing herbivores as bark lice (*psocids*: Turner 1975) or infection with the basidiomycete *Athelia epiphylla* (Poelt and Jülich 1969). Furthermore, mutualistic or even symbiotic interactions with heterotrophic partners constitute another competitive strategy which has not been investigated so far. Generally, it is stated that organisms dominating a challenging environment exist on such a "tight budget" (Nienow and Friedmann 1993), that antibiosis is rarely observed (Friedmann and Ocampo-Friedmann 1984, Gorbushina et al. 2005) and consequently symbiosis is an essential advance of survival strategies. However, to solve these open questions, the unique feature(s) separating *Apatococcus* from other aeroterrestrial green algae need to be highlighted by comparative studies taking all the discussed variables and possibilities into account.

Biochemical adaptations. While morphological structures avoid or delay water loss, the biosynthesis and accumulation of *organic compounds* known as "compatible solutes" further contribute to desiccation tolerance (Karsten et al. 2007, Gustavs et al. 2009a). The underlying compounds include polyols, sugars and heterosides, amino acids and derivatives, as well as quaternary amines such as glycine betaine (Yancey 2005). Organic osmolytes are typically low-molecular weight carbohydrates (LMWCs), soluble at high concentrations in water, and either uncharged or zwitterionic at physiological pH-values. The compatible solutes exert multiple functions in metabolism, that is besides their role as organic osmolytes and compatible solutes, they can also act as antioxidants, heat protectants (stabilization of proteins) and rapidly available respiratory substrates (Eggert and Karsten 2010 and references therein). The presence of polyols in Trebouxiophyceaen green algae has been reported

within this study (Gustavs and Karsten, submitted). Additionally, the function of ribitol as organic osmolyte has been demonstrated for the first time in free-living aeroterrestrial algae (Gustavs et al. 2009b). It is assumed that the occurrence of polyols in the vaste majority of the *Trebouxia*-lineage is a general adaptation to the terrestrial (sometimes cosmopolitan) lifestyle (Darienko et al. 2010). Small organic compounds can further act as anti-freezing agents in the cytoplasm. Particularly the amino acid proline and sulfonium compounds such as dimethylsulfoniumpropionat (DMSP) serve as anti-freezing agents in polar micro- und macroalgae (Bartsch 1989, Nishiguchi and Somero 1992, Karsten et al. 1996). During desiccation, trehalose binds to membranes by replacing water and maintaining their basic structure. It builds a glass-like state under dry conditions contributing to the preservation of cellular structures (Yancey 2005 and references therein).

Changes in *pigment* composition and concentration in response to stressful conditions have also been reported frequently. Tjahjono et al. (1994) described a significant enhancement of astaxanthin production in *Haematococcus* due to a temperature increase from 20°C to 30°C. Liu and Lee (2000) confirmed that observation for the green alga *Chlorococcum* sp., as the carotenoid concentration increased two-fold due to a temperature enhancement from 20 to 35°C. It is speculated that enhanced temperatures force the building of reactive oxygen species (ROS) which in turn is compensated by an increased carotenoid concentration trapping free radicals (Tjahjono et al. 1994). Excessive radiation leads to decrease of Chl *a* concentration and the increase of carotenoids and xanthophylls. Primary carotenoids are located in the thylakoid membranes contributing to photosynthetic activity and photoprotection. The over-accumulation of secondary carotenoids in lipid vesicles in the chloroplast stroma or in the cytosol is well known for Chlorophyceae such as *Dunaliella* or *Haematococcus* (del Campo et al. 2007). This phenomenon is discussed as a reaction to environmental stresses such as excessive irradiance, nitrogen deficiency or osmotic stress. It also seems to be characteristic for the Oocystis-lineage within the Trebouxiophyceae (T. Pröschold, pers. communication).

Mycosporine-like amino acids (MAAs) constitute protection against harmful UV radiation, efficiently absorbing wavelengths around 320-340 nm thus enabling microorganisms to colonize terrestrial habitats. Karsten et al. (2007) investigated the effect of UVR (8 Wm^{-2} UVA and 0.4 Wm^{-2} UVB) on photosynthetic efficiency, growth rate and MAA concentration of *Stichococcus* spec. and *"Chlorella" luteoviridis* in comparison to the aquatic *Desmodesmus subspicatus*. UVR exposure led to only minor effects on growth and photosynthesis in the MAA-containing aeroterrestrial isolates, indicating effective photoprotection, while the aquatic species was strongly affected.

Conclusions and directions for future work

The discussed interdisciplinary project revealed detailed knowledge about species diversity in aeroterrestrial green biofilms throughout Germany which, however, has been underestimated significantly by traditional microscopical identifications. Furthermore, phylogenetic analysis of obtained sequence data led to an improved understanding of Trebouxiophyceaen taxonomy. The ***species concept*** for green microalgae is in a phase of redefinition and the scientific community recommends polyphasic approaches combining as many different datasets as possible. Therefore, the reassessment of phylogenetic status of *Chloroidium* by such an approach is of great importance and effectively demonstrates the potential of interdisciplinary research. Further, the classification of "*Chlorella*-like" algae in general is urgently needed, as the phylogenetic diversity within this morphological cluster is well-known and a definite species assignment is a mandatory prerequisite for interpretation of scientific data. However, *Chloroidium* is an interesting species in its own right, as it is a cosmopolitan representative competing successfully in marine, limnic and terrestrial habitats. Both biogeographical distribution and regulatory mechanisms concerning wide tolerance limits regarding many abiotic factors (crucial for cosmopolitan appearance) are interesting future research fields. However, the concept of ***chemotaxonomy*** is a promising approach supporting investigations about green algal taxonomy and biogeography, as it allows differentiation between morphological similar species as *Apatococcus* and *Desmococcus* or *Chloroidium* and *Chlorella*. The Trebouxiophyceae are of polyphyletic origin and can be partitioned into the Trebouxia-, Chlorella- and Oocystis-lineage which seem to be biochemically separated through accumulation of polyols, ergosterol and secondary carotenoids, respectively. The latter observation needs systematic investigation, as green algae belonging to the Oocystaceae have not been involved in systematical screening so far. Nevertheless, the synthesis and accumulation of polyols is assessed as a major ***adaptation strategy*** to the aeroterrestrial habitat, enabling the algae to withstand water limitation which is regarded as the key factor for algal growth. Consequently, the ability to take up water vapor and to grow without liquid water is a crucial competence for survival of respective green algae. It has been shown that aeroterrestrial green algae are euryoecious concerning temperature and radiation ranges and display effective photoprotective mechanisms. *Apatococcus* has been identified as the most abundant aeroterrestrial green alga and is regarded as optimally adapted to its environment. Besides mixotrophic metabolism and the general ability to survive long periods of unfavorable conditions, positive ***species interaction***s within the biofilm assemblage are considered as explanation for its ecological success. *In situ* measurements of metabolic activity within biofilms as well as investigations concerning mutualistic or symbiotic relationships will contribute to a deeper understanding of its competitive strength. Thus, this alga is considered an optimal model organism for

future research on biofouling prevention and furthermore an interesting representative of aeroterrestrial microalgal community.

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6.2 Erklärung über den geleisteten Eigenanteil an den wissenschaftlichen Veröffentlichungen bzw. Manuskripten

Die vorliegende Doktorarbeit besteht aus drei wissenschaftlichen Veröffentlichungen, einem eingereichten Manuskript sowie einer Publikation in Vorbereitung.

1. Darienko T, **Gustavs L**, Mudimu O, Rad-Menendez C, Schumann R, Karsten U, Friedl T and Pröschold T (2010) *Chloroidium*, a common aerophytic coccoid green alga previously assigned to *Chlorella* (Trebouxiophyceae, Chlorophyta). *Eur. J. Phycol.,* DOI 10.1080/09670260903362820
    - Datenerhebung 30%
    - Datenauswertung 30%
    - Manuskript 40%
2. **Gustavs L** and Karsten U (2010, submitted) Polyols as chemotaxonomical markers to differentiate between green algae (Trebouxiophyceae, Chlorophyta). *J. Phycol.*
    - Datenerhebung 90%
    - Datenauswertung 90%
    - Manuskript 90%
3. **Gustavs L**, Schumann R, Eggert A and Karsten U (2009) *In vivo* growth fluorometry: Accuracy and limits of this non-invasive and simple method to measure growth of microalgae. *Aquat. Microb. Ecol.,* **55**: 95–104
    - Datenerhebung 60%
    - Datenauswertung 70%
    - Manuskript 70%
4. **Gustavs L**, Eggert A, Michalik D and Karsten U (2009) Physiological and biochemical responses of green microalgae from different habitats to osmotic and matric stress. *Protoplasma*, DOI 10.1007/s00709-009-0060-9
    - Datenerhebung 90%
    - Datenauswertung 90%
    - Manuskript 80%
5. **Gustavs L**, Schumann R, Polerecky L, Lorenz M and Karsten U (in prep.) The role of mixotrophy in metabolic performance of *Apatococcus lobatus* (Trebouxiophyceae, Chlorophyta), an abundant aeroterrestrial green alga.
    - Datenerhebung 80%
    - Datenauswertung 80%
    - Manuskript 80%

6.3 Erklärung der Selbstständigkeit

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Lydia Gustavs Rostock, den 09. April 2010

6.4 Lebenslauf

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